University of Alberta

Enhanced Laboratory-based Surveillance of Enteric Virus in Children in Northern Alberta

by

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Dedication

To my husband Brian Wong and to my nanny 好姐

Abstract

During a 28-month period, 1,927 stool specimens from children younger than seven years submitted to Provincial Laboratory for Public Health for virological investigations were tested by nucleic acid amplification tests (NAT) for rotavirus, enteric adenovirus, astrovirus and norovirus. Compared to electron microscopy, NAT increased the detection of rotavirus by 29%, adenovirus 8%, astrovirus 62% and norovirus 98%. After removing duplicate and discordant specimens, the frequency of each virus (N=1,732 cases) was: rotavirus (22%), norovirus (10%), astrovirus (4%) and adenovirus (3%). Forty-one cases of mixed infections were detected only with NAT. Rotavirus and norovirus infections were associated with young age. Mistahia and Northern Lights regions were associated with a higher disease burden of rotavirus and adenovirus respectively. Rotavirus, norovirus and astrovirus showed predominance in the cold weather while enteric adenovirus had the lowest prevalence in the Winter. Enhanced laboratory-based surveillance of enteric virus in Northern Alberta was achieved with NAT.

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List of Abbreviations

bp	Base-pair
Ct	Threshold cycle
Df	Degree of freedom
EIA	Enzyme immunoassays
EM	Electron microscopy
NAT	Nucleic acid amplification test
ORF	Open reading frames
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
ProvLab	Provincial Laboratory for Public Health
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
sp	Species
SRSV	Small round structured virus
Tm	Melting temperature

CHAPTER 1 BACKGROUND

1.1 Disease burden of gastroenteritis

Gastroenteritis is a common childhood illness that results in significant morbidity and mortality. In low-income countries, approximately 3.3 million deaths occur each year from gastroenteritis with the mortality rate highest in the first year of life at about 20 deaths per 1,000 children.[1] Even though the mortality rate in children between one and four years of age is lower at five deaths per 1,000, this age group still accounts for about half of the deaths due to gastroenteritis in childhood. In the Global Burden of Disease study, causes of death were divided into nine clusters and three groups by various characteristics and gastroenteritis was classified in the group of communicable, maternal, perinatal and nutritional disorders.[2] According to this study, while mortality due to gastroenteritis was projected to fall from 2.9 million in 1990 to 1.5 million in 2020, gastroenteritis would still remain as one of the 10 leading causes of death worldwide.

Mortality from gastroenteritis is lower in high-income countries as compared to low-income countries, yet it is still an important problem in high-income countries because of the high disease burden. In United States, the averaged child will have had 7-15 episodes of gastroenteritis by the age of five years, resulting in 2-3.5 million doctor visits, greater than 200,000 hospitalizations, and 325-425 deaths with approximately 65% of the hospitalizations and 85% of the diarrheal deaths occurring in the first year of life.[3]

From a study in greater Toronto / Peel region in Ontario, the rate of hospitalization of children younger than five years for gastroenteritis was 4.8 per 1,000 in a 12-month period with a mean ± standard deviation (SD) stay of 3.1 ± 1.6 days.[4] In another study on gastroenteritis in outpatient settings and child care centers in the same geographic area, the rates of diarrhea per 100 children per month were 6.6 episodes in young children aged between 0 to 23 months, 1.9 episodes for those children between 24 to 35 months of age and 0.07 episodes for those aged three years and older during an eight-month study period.[5]

1.2 Etiology of childhood gastroenteritis

It is important to know the etiology of gastroenteritis in order to understand disease burden and planning of disease control strategies, especially in terms of vaccine development. In the US, enteric viruses are the leading cause of gastroenteritis in young children.[6] From a worldwide perspective, in 1991, the World Health Organization Diarrhoeal Disease Control Programme published a two year surveillance study for childhood gastroenteritis in China, India, Mexico, Myanmar and Pakistan using standardized laboratory diagnostic protocols. In those five countries, rotavirus and two enteric bacteria: *Shigella* spp. and enterotoxigenic *Escherichia coli* were the three pathogens most strongly associated with disease.[7] Upon reviewing of the literature of clinic-based or laboratory-based surveillance studies that tested for enteric bacteria, parasites as well as enteric viruses in children with gastroenteritis, enteric viruses, and

especially rotavirus were the leading pathogens in gastroenteritis in young children in all the studies (Appendix A).[8]

1.3 Enteric viruses

1.3.1 Historical background

The diagnostic abilities to detect viruses in stool samples led to the recognition of these infectious disease agents as important etiological agents in gastroenteritis. Kaipankian et al. were the first to provide evidence that norovirus, (initially known as Norwalk virus) was the cause of a gastroenteritis outbreak in Norwalk, Ohio.[9] Rotavirus, enteric adenovirus and astrovirus were subsequently identified a few years later as causative agents by fulfilling criteria of Koch's postulate including: 1) measurable immune response to the agent, 2) presence of the agent more commonly in cases versus controls, 3) logical time frame of the detection of the agent and onset of illness, and 4) infectivity of fecal filtrates with the agent in human or animal studies. Other novel viruses such as torovirus and coronavirus have been identified in stool samples, yet more evidence is required before acceptance of these agents as causative agents of human gastroenteritis.[10]

The viruses that are proven causes of gastroenteritis fall into four distinct families – rotavirus (*Reovridae*), enteric adenovirus (*Adenoviridae*), astroviruses (*Astroviridae*) and human calicivirus [norovirus and sapovirus] (*Caliciviridae*). Among the groups, rotavirus is a non-enveloped doubled stranded RNA virus; norovirus and astrovirus are non-enveloped single-stranded, positive-sense RNA virus and enteric adenovirus is a non-enveloped DNA virus. Another way to

characterize these viruses is based on the setting from which they have been most commonly identified as causative agents. For example, norovirus is the most common cause of food-borne and gastroenteritis outbreaks whereas rotavirus, astrovirus and enteric adenovirus are mainly identified as causes of sporadic endemic gastroenteritis.

1.3.2 Rotavirus

Rotavirus is characterized by a 'wheel-like' image (70-nm diameter) under electron microscopy (EM). The viral capsid has a double protein layer with the outer capsid made of structural proteins VP7 and VP4 and inner capsid mainly of VP6. The viral genome contains 11 segments of double stranded RNA that encodes the various structural proteins (VP) and non-structural proteins (NSP). Rotavirus is classified according to the antigenic properties of the various capsid proteins into: 1) groups (A to G, by VP6 protein), 2) subgroups (I and II, by VP6 protein), and 3) serotypes (G and P types by VP7 and VP4).[11] Group A, B and C can cause human infections with group A and serotypes G1-G4 accounting for 95% of clinical illness.[12]

Rotavirus is highly infectious with incubation ranging from one to seven days (usually less than two days). Clinical infection can occur with exposure as little as 10^4 to 10^5 particles. Infection can be asymptomatic or be associated with severe vomiting and profuse diarrhea. A rare syndrome with central nervous system involvement that might be due to direct viral invasion had been reported.[13,14] Oral rehydration and supportive therapy is the mainstay of

management. In temperate climates, rotavirus disease usually peaks in the winter months with seasonality less marked in tropical regions.

While the immunological response to rotavirus infection has not been fully characterized, most of the infections occur before two years of age with fewer symptomatic infection with time.[15,16] The presence of protective immunity against subsequent infections forms the basis of vaccine development against this enteric virus that has a major impact on global gastroenteritis burden.

A review by the Centers for Disease Control and Prevention of studies published from 1986 to 2000 on deaths caused by diarrhea and rotavirus infections in children estimated an global annual incidence of 111 million episodes of rotavirus gastroenteritis that required only home care, 25 million clinic visits, two million hospitalizations and 352,000 - 592,000 deaths (median, 440,000) in children less than five years of age.[17] Children in low-income countries account for 82% of rotavirus deaths.

1.3.3 Enteric adenovirus

Human adenovirus comprises 49 distinct serotypes that are grouped into six subgenus based on various immunological and biological characteristics.[18] The viral capsid is icosahedric, 70 nm in diameter, and is comprised of 252 protein capsomers and structures called fibres that protrude to the outside providing a characteristic appearance under EM. The viral DNA encodes nine transcription units: six that are expressed early after infection (E1A, E1B, E2A, Es, E4 and L1) and three that are activated as intermediate (pIX and IVa2) and one as late (major late transcription unit {MLTU}) expression during the infection.

Most gastroenteritis is caused by adenovirus serotypes 40 and 41 which belong to subgenus F.[9] Many infections with adenovirus are asymptomatic, especially in cases where neutralizing antibodies have been induced with prior infections. The incubation and duration of gastroenteritis is usually longer than other enteric viruses.[11]

1.3.4 Astrovirus

Astrovirus was named for its distinctive star-like appearance on EM (astron 'Greek' = star). This is a small round virus (28-41 nm) depending on the visualization of protein spikes on the viral capsid.[19,9,20] Only 10% of the viral particles have the characteristic star-appearance by EM and thus the virus is usually only reported as small round structured virus (SRSV) by EM. There are eight reported serotypes of human astrovirus, with serotype 1 being the most prevalent. The RNA genome contains three open reading frames (ORF), designated as ORF1a, ORF1b, and ORF2, that encode both structural and non-structural proteins.

The peak seasons of astrovirus infections are during the Winter in temperate climates and during the rainy season in tropical climates. The incubation period is between three to four days in the studies of human volunteers and between 24-36 hours when extrapolated from outbreak investigations. The disease typically lasts for three to four days, and is less severe as compared to rotavirus infection.[21]

1.3.5 Calicivirus

Because of its important role in gastroenteritis outbreaks, within the *Caliciviridae* family, norovirus is more studied and described compared to sapovirus.[22,23] Norovirus has long been recognized as a causative agent in food borne gastroenteritis outbreaks especially via shell fish in coastal areas. Recently, norovirus has also acquired notoriety as a major cause of outbreak on cruise ships.[24,25] In Alberta, norovirus is the most common identifiable cause of gastroenteritis outbreaks (up to 60-70% of outbreaks) in long term care facilities and sometimes in hospital setting (data not shown).

On EM, norovirus is usually reported as SRSV as the particles are small (35-39 nm) with few distinguishable features; unlike other caliciviruses, which may exhibit a 'Star of David' structure.[11] The RNA genome has three major ORFs that encode the structural and non-structural proteins. Based on the comparisons of genetic sequences of the viral RNA-dependent RNA polymerase and the capsid protein, noroviruses are subdivided into five genogroups (GI – GV). Genogroup I with seven clusters and genogroup II with 16 clusters contain most of the strains infecting humans. The genetic diversity of norovirus and the absence of an *in vitro* culture system contributed to the difficulty in the characterization of the virus in the early days. With the cloning of the virus and development of nucleic acid amplification test (NAT), i.e., detection based on the presence of genetic materials (nucleic acids) using technology such as polymerase chain reaction (PCR), norovirus is increasingly being recognized as an important cause of sporadic gastroenteritis in children.[26,27,28,29]

Norovirus infection is sometimes referred to as 'Winter vomiting illness' reflecting the observation that vomiting is often the first presenting symptom. Incubation period is usually short, 24-48 hours. In a natural history study, diarrhea as a symptom was more prevalent in children less than one year of age and a greater proportion of children aged between five and 11 years had vomiting compared to the younger children.[30] In the same study, the duration of illness was longer than the previously described three to five days with a median length of illness of five days and a shorter median duration of illness with increasing age. One of the most interesting advances in the understanding of the pathogenesis of norovirus comes from the studies on the differences in susceptibility to infection and host factors such as ABO blood group antigens.[31,32]

1.4 Diagnostic methods for enteric virus

Isolation and identification of specific viral pathogens in gastroenteritis has been limited by the lack of simple and sensitive diagnostic tests.[10] EM has been the standard conventional diagnostic approach to enteric viruses since the 1970s. While EM is good at detecting various enteric viruses just by examining a stool sample, this technology is limited because: 1) there is a requirement for technical expertise and expensive instrumentation for the diagnostic laboratory, 2) it has low sensitivity: the limit of detection using EM is estimated to be 10^6 viral particles per gram of stool, and 3) it has low specificity: of the four enteric viruses, only rotavirus and adenovirus can be easily identifiable by EM while astrovirus and norovirus can only be reported as SRSV because of the small size and the absence of distinguishable features.[11,19,33,34,35,36]

With better characterization of the enteric virus, commercial enzyme immunoassays (EIA) using antibodies specific for each virus, e.g., rotavirus, enteric adenovirus and astrovirus have been developed. While some studies report equal or better performance of the EIA when compared to EM for these viruses, other studies have reported low sensitivity and specificity of EIA assays.[37,38,39,40,41,42,43,44,45,46] Of note, the performance of EIA assays has been poor for norovirus because of its genetic and antigenic diversity.[47]

Since the 1990s, PCR-based techniques have been used to detect and genotype viruses in stool specimens in various laboratories as research studies. For example, molecular methods utilizing reverse transcriptase PCR (RT-PCR) have increased the rate of detection of rotaviruses by 15 to 27% in comparison to EIA.[48,49,50] Similarly for astrovirus and norovirus, cloning and sequencing has allowed for the development of broadly reactive molecular detection assays.[51,52,53,54,26,27,28,29] However, all these studies have focused on one specific viral agent. The first large scale population-based surveillance studies of several enteric viruses using NAT assays were performed in Finland.[21,55,56,57,58,59] The RT-PCR and PCR assays used in Finland identified viral etiologies in 60% of all episodes of acute community-acquired gastroenteritis in young Finnish children and 85% of the moderately severe and severe cases. Interestingly, norovirus was found to be as common as rotavirus in community-acquired acute childhood gastroenteritis, with each virus detected in 20-30% of the stool specimens.

Another technical advancement since the completion of these studies is the development of molecular diagnostic platforms for real-time NAT. Real-time NAT is a technological advancement with many applications in the molecular diagnostics field.[60,61] It also has many advantages including greater accuracy, sensitivity, specificity, faster turn-around time, the ability to quantitate and the minimization of cross-contamination as a result of a close-tube system.

1.5 Northern Alberta data

Viral etiologies for acute gastroenteritis in young children in Northern Alberta remain poorly characterized. Prior to the current study, from January 2000 to January 2001, a total of 1,156 stools specimens were processed for EM and viral studies at the Provincial Laboratory for Public Health (ProvLab) (unpublished data). The majority of these specimens (57%) were collected from children less than three years of age. Forty-seven (4.1%), 61 (5.3%) and 259 (22.4%) of the specimens were collected from children and adolescents between the ages of three to six years, seven to 20 years and 70 to 100 years respectively. The 70 to 100 year old age group was likely residents of long-term care facilities who were being investigated for gastroenteritis outbreaks. The rest of the specimens were quite evenly distributed in 10-year-age groups with 16 to 41 specimens (1.4 to 5.3%) per age group. EM detected enteric viruses in only 5-15% of the stool specimens depending on the age group.

1.6 Objectives

1. To use the nucleic acid amplification testing (NAT) method to detect the four common viral pathogens of childhood gastroenteritis: real-time RT-PCR for rotavirus and norovirus, real-time PCR for enteric adenoviruses, and conventional RT-PCR for astrovirus, and to compare the utility of the NAT assays with traditional methods (EM and viral culture) used in the laboratory in the diagnosis of viral gastroenteritis.
2. To describe the factors associated with viral gastroenteritis including age and gender of the patients, health regions, and medical specialty of submitting physicians.
3. To review the seasonality of these enteric viruses.

1.7 Hypothesis

The hypothesis of the current study is that NAT is more sensitive as compared to EM and culture and will result in a significant increase in the detection and identification of enteric viruses in childhood gastroenteritis. We also hypothesize that, similar to studies in Finland, rotavirus will be the most common pathogen in young children in Alberta and that the seasonal distribution of these virus in Northern Alberta will be similar to that previously described in temperate regions.

CHAPTER 2 METHODS

2.1 Routine virological investigations of stool samples at Provincial Laboratory

All the requests for viral studies of stool specimens in Northern Alberta are referred to the Provincial Laboratory for Public Health (Microbiology) (ProvLab), Edmonton site, for electron microscopy (EM) and/or viral culture. Using the health region designations used by Alberta Health and Wellness from May 2001 to April 2003, specimens were submitted to ProvLab, Edmonton site, mainly from 12 of the 17 regions in Alberta: Northwestern Health Region (region 17), Northern Lights Health Region (16), Keeweetinok Lakes Health Region (15), Peace Health Region (14), Mistahia Health Region (13), Lakeland Health Region (12), Aspen Health Region (11), Capital Health Region (10), Crossroad Region (9), Westview Region (8), East Central Health Region (7), and David Thompson Region (6). A map showing all 17 regions in Alberta as designated during May 2001 to April 2003 is in Appendix B. On occasion, referral specimens were also sent from the Northwest Territories, Nunavut, Yukon and Saskatchewan.

Prior to July 21, 2003, EM and viral culture were routinely performed on the stool specimens if they were collected from children under the age of three years. Stool specimens collected from patients aged three years or older would be processed for EM and viral culture if there was a specific request for viral investigations on the requisition. The testing algorithm for enteric virus at ProvLab was changed on July 21, 2003 to only performing EM on the stool

specimens unless there was a specific request for culture for enterovirus or coxsackie virus, as most of the enteric viruses are not identifiable by routine viral culture.[22]

2.2 Study population

2.2.1 Phase I study population

The inclusion criteria were stool specimens submitted from individuals less than seven years of age to ProvLab, Edmonton site, from September 1, 2001 to August 31, 2002 for EM and/or viral culture. Aliquots of the specimens were made and stored at –20°C before being screened by nucleic acid amplification tests (NAT) for enteric adenovirus, rotavirus, astrovirus and norovirus.

2.2.2 Phase II study population

The inclusion criteria were stool specimens submitted from patients to ProvLab from Jan 1, 2003 to April 30, 2004 for viral studies, including EM and/or viral culture. These specimens were stored at –20°C and aliquots were later made of available specimens from patients less than seven years of age and stored at –20°C before being tested by three NAT assays for rotavirus, astrovirus and norovirus.

2.3 Extraction of nucleic acids

2.3.1 Extraction in Phase I

Stool specimens stored at –20°C were weighed and suspended in 10% weight by volume phosphate-buffered saline (PBS), vortexed and subsequently

centrifuged at 4°C for 10 minutes. Viral RNA was extracted from 100 μl of 10% stool suspension by the guanidinium isothiocyanate method and purified by size-fractionated silica as previously described.[62] The silica adsorbed with RNA was pelleted, washed and dried at 50°C for 10 minutes. The RNA was eluted from the silica and stored at –70°C until being used in NAT.

2.3.2 Extraction in Phase II

The procedure used in phase II is similar to phase I except that stool specimens stored in –20°C were weighed and suspended in 20% weight by volume PBS, vortexed and subsequently centrifuged at 4°C for 10 minutes. Viral RNA was extracted using Magazorb™ RNA extraction kit (CORTEX Biochem, CA, USA) according to the manufacturer's instructions, and 100 μl RNA was eluted from 200 μl of 20% stool suspension.

2.4 Nucleic acid amplification (NAT)

RT-PCR and PCR assays were carried out in separate rooms for template preparation, PCR assay mix preparation, and PCR product analysis respectively. All rooms were kept clean and UV-sterilized daily to avoid contamination. Two negative controls and positive controls were used in each patch of RNA or DNA extraction, and two negative controls (water) in each PCR run.

2.5 Specific PCR and RT-PCR assays

2.5.1 Assay for Rotavirus

For both phase I and phase II of the study, all the stool samples were tested with a real time quantitative TaqMan RT-PCR assay that was developed and validated during the study. For TaqMan Real time RT-PCR, the PCR primers were selected from a highly conserved region of the group A rotavirus non-structural protein 3 (NSP3) sequence (Appendix C). The size of the expected amplicon was 87 base-pair (bp). The PCR primers were selected from a highly conserved region of the group A rotavirus non-structural protein 3 (NSP3) sequence (Appendix C).

A single-step real time RT-PCR reaction using commercialized TaqMan EZ RT-PCR Core Reagent Kit (Applied Biosystems, CA, USA) was carried out with an ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described.[63] The fluorogenic probe was labelled with a FAM reporter at the 5' end and a TAMRA quencher at the 3' end. A single step real time RT-PCR reaction using commercialized TaqMan EZ RT-PCR Core Reagent Kit (Applied Biosystems, CA, USA) was carried out with an ABI Prism 7700 Sequence Detection System (Applied Biosystems).

RT-PCR reaction was performed in 25 μl volume containing 5 μl of 5x TaqMan EZ buffer, 3mM $MnCl_2$, 300 μM each of dATP, dCTP, dGTP and dUTP, 2.5 unit rTth DNA polymerase, 0.25 unit AmpErase UNG, 200 nM of each primer, 150 nM of probe, and 5 μl RNA. AmpErase UNG was added to prevent reamplification of carryover contaminations.

After initial incubation to inactive uracil-N-glycosylase at 50^0C for two minutes according to the manufacturer's instruction, reverse transcription was performed at 60^0C for 30 minutes, followed by two-step thermal cycles of 94^0C for 20 seconds and 60^0C for 1 minute by 45 cycles after re-heating at 95^0C for 5 minutes. Real time measurements were taken and a threshold cycle (C_t) value for each sample was calculated by determining the point at which the fluorescence exceeded a threshold limit of 0.04 with a FAM reporter at the 5' end and a TAMRA quencher at the 3' end.

2.5.2 Assay for enteric adenovirus

Only the specimens from the phase I study were tested by a real time LightCycler DNA PCR assay that was developed during the study and the assay was specific for enteric adenovirus 40 and 41. For quantitative LightCycler Real time PCR for enteric adenovirus, the primers and probes were selected from the Ead genetic sequence (Appendix C).

The size of the expected amplicon was 187 bp. The LightCycler assay was developed and validated using a previously described LightCycler PCR assay.[64] The PCR primers were designed using LightCycler probe design software (Roche Diagnostics) from human adenovirus type 41 hexon gene expected to yield a 188 bp product by PCR amplification. The specific hybridization donor probe labelled with fluorescein at the 3' end and the acceptor probe labelled with LC-Red 640 at the 5' end were used for real-time detection during the LC-PCR reaction. All primers and probes were purchased from TBI Molbiol LLC (New Jersey, USA).

Twenty μl of the PCR reaction mixture containing 5 μl DNA solution, 4 mM $MgCl_2$, 0.4 μM of each primer, 0.2 μM of each probe, and two μl of the reagent from a LC-FastStart DNA Master hybridization probe kit (Roche Diagnostics) were added to a capillary tube (Roche Diagnostics). The capillary tubes were centrifuged, mounted onto the carousel and loaded into the LightCycler.

The thermal cycles were as follows: an initial 10 minutes at 95^0C, followed by 45 cycles of 5 sec denaturing at 95^0C, 10 sec annealing at 56^0C, and 10 sec extension at 72^0C. The data were collected in the single mode with channel setting F2 / F1 during the annealing phase.

For data analysis, the baseline was adjusted using the arithmetic mode and the fluorescence curve fit performed in the fit point mode with two points of the LC software (version 3). Specimens with a fluorescence signal higher than the background were positive. Water was used as negative controls in both DNA extraction and DNA amplification.

2.5.3 Assay for astrovirus

Specimens from both phase I and phase II of the study were tested by a conventional RT-PCR assay for astrovirus. For Conventional RT-PCR for astrovirus, the set of primers, Ast-beg, was selected for the amplification reaction (Appendix C).[65]

RT-PCR was performed as previously described.[21] The RT-PCR procedure for astrovirus was modified from Myers et al.[66] Briefly, two μl extracted RNA was added to 8 μl RT reaction mixture containing 1 μl RT 10x buffer, 1mM $MnCl_2$, 1 μl dNTP mix, 10 unit inhibitor and 6 unit Tth DNA

Polymerase and 0.5 mM each primer Beg and End. The reaction mixture was incubated at 80°C for 3 minutes, then at 60°C for 20 minutes and cooled on ice for 5 minutes. Forty µl PCR reaction mixture containing 4 µl chelated-buffer (Promega, Madison, WI, USA), 3 µl 25mM $MgCl_2$, and 3 µl dNTP were added to the RT reaction mixture for PCR reaction.

The reaction mixture was incubated at 94°C for 3 minutes, and run 30 cycles of 45 seconds at 94°C, 1 minute at 55°C, 1.5 minutes at 72°C, with a final extension of 7 minutes at 72°C. One ml of first PCR product was transferred to run the nested PCR. The cycling conditions were identical to those in the first PCR. The expected amplicon size was 241 bp and the PCR products were separated in 3% agarose gel and visualized by ethidium bromide staining.

2.5.4 Assay for norovirus

For the phase I study, a conventional RT-PCR was used to screen the stool samples for norovirus and positive results were confirmed by a real time LightCycler RT-PCR assay developed during the study. For the phase II study, all the stool samples were tested with a real time TaqMan RT-PCR assay also developed during the study.

2.5.4.1 Primers for the real time LC RT-PCR and conventional RT-PCR

The same set of primers were used for the real time LC RT-PCR and conventional RT-PCR. Primers selected from a conserved region of norovirus polymerase gene, NVP 110, were used in the detection of norovirus GII and for

the detection of norovirus GI, the primers from the capsid gene of norovirus CapA were used.[67] (Appendix C)

2.5.4.2 RT reaction

RT reaction was carried out with SuperScript™ II RNase H⁻ Reverse Transcriptase kit (Invitrogen, CA, USA). A final volume of 20 μl RT reaction containing 5 μl of 5x first transcript buffer, 5 mM DTT, 20 unit RNaseOut™ recombinant ribonuclease inhibitor, 100 unit SuperScript™ II Reverse Transcriptase, 2.5 mM each of dATP, dCTP, dGTP and dTTP, 1.25 μM primer NVP110 or primer CapA, and 5 μl RNA (equivalent two mg stool) was incubated at 45°C for 1 hour, then inactivated at 70°C for 15 minutes.

2.5.4.3 Conventional PCR

A final volume of 50 μl of reaction containing 5 μl of cDNA (equivalent to 0.5 mg of stool) from the RT reaction, 5 μl of 10x PCR Buffer, 2 mM of $MgCl_2$, 0.375 mM each of dATP, dCTP, dGTP and dTTP, 0.5 μM of each primer, 2.5 μl AmpTaq polymerase (PE Biosystem, CA, USA) was carried on GeneAmp PCR System 9600 (Perkin Elmer). The reaction was preheated at 95°C for 3 minutes and followed by 40 thermal cycles of 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C, and a final extension at 72°C for 5 minutes. The PCR products were separated in 3% agarose gel and visualized by ethidium bromide staining.

2.5.4.4 Quantitative Real time LightCycler RT- PCR (LC RT-PCR)

A SYBR green I system was utilized in the reaction. Twenty μl of the PCR reaction mixture containing 2 μl cDNA (equivalent to 0.2 mg of stool) from the RT reaction, 3 mM $MgCl_2$, 0.5 μM of each primer, 2 μl of the reagent from a LC-FastStart DNA Master SYBR green kit (Roche Diagnostics) were added to a capillary tube and loaded into the LightCycler (Roche Diagnostics).

The thermal cycling conditions were as follows: an initial denaturation at 95°C for 6 minutes, followed by 45 cycles of 10 sec denaturing at 95°C, 10 sec annealing at 50°C, and 10 sec extension at 72°C. The data were collected in the single mode with channel setting F1 / 1 during the annealing phase. To establish external standard curves for the quantification of norovirus, RNA transcripts from strains S5 and S19 corresponding to G-I/4 and G-II/12 respectively (provided by Dr. T. Ando, CDC, Atlanta) were ten-fold diluted (5 to $5x10^6$ copies per reaction) and run in real time LC RT-PCR reaction. To determine potential contamination from untranscribed plasmid DNA in the RNA preparations, the same serial dilutions were tested using real time LC PCR reaction without the RT reaction.

For data analysis, melting temperature (Tm), fluorescence-dF1/dT under melting curve window and Ct, which is defined as the fractional cycle number where the fluorescence passed the fixed threshold, in quantification window were selected as the evaluating parameters. Readout of the reaction with Tm of 82 to 85°C, fluorescence-d[F1]/dT above 1.5, and Ct value below 38.00 against a baseline of fluorescence signal at 2.0 was used to indicate a positive reaction.

2.5.4.5 Quantitative Real time TaqMan RT-PCR

The primers and probes were selected from norovirus ORF1-ORF2 junction region where the highest homology of sequences exists.[68] Norovirus GI and GII probes labelled with TaqMan dye VIC and FAM respectively were synthesized by Applied Biosystems.[69] The forward and reverse primers at 300 nM were used in the RT reaction with SuperScript™ II RNase H⁻ Reverse Transcriptase kit (Invitrogen, CA, USA).

A final volume of 20 μl RT reaction containing 5 μl of 5x first strain transcript buffer, 5 mM DTT, 20 unit RNaseOut™ recombinant ribonuclease inhibitor, 100 unit SuperScript™ II reverse transcriptase, 2.5 mM each of dATP, dCTP, dGTP, and dTTP, 300 ng random primer, and 5 μl RNA was incubated at 42°C for 1 hour, then inactivated at 70°C for 15 minutes. Mrt TaqMan PCR reaction was performed in 25 μl volume containing 12.5 μl of Universal DNA Master Mix (Applied Biosystems), 5 μl cDNA, 400 nM each GI and GII primers, 200 nM each GI and GII probes. After initial incubation at 50°C for 2 minutes to activate UNG and then at 95°C for 10 minutes for denaturing, PCR amplification was performed with two steps thermal cycles of 94°C for 20 seconds and 56°C for 1 minute by 45 cycles after re-heating at 95°C for 5 minutes. Amplification data was collected and analyzed with Sequence Detection Software version 1.0 (Applied Biosystems).

2.6 Demographic data

The date of specimen collection when available, or otherwise, the date of receipt of the specimen was used to characterize the monthly distribution of

samples. Age and gender of the patient, name of the submitter and the health region assigned to each specimen were also documented. Submitters were categorized into four different types of practice: 1) general pediatricians or pediatric specialists including surgeons, 2) family physicians or general practitioner, 3) medical officer of health or nurse-in-charge and 4) unclassified. For assignment of the health region, if a patient's address was available on the requisition, the health region of the patient's residence was used for the assignment. Otherwise, the health region of the submitter, followed by the health region of the submitting location in the order of priority was used to determine this designation. The respective number of specimens that had the region assigned by patients' residence, submitters or agencies was not documented in phase I; for the 1,509 specimens from phase II, 31.5% were designated by patients' residence, 51.4% by physicians' regions and 17.0% by submitting agencies.

Geographic regions of the specimens were reclassified into five groups to analyze the distribution of the specimens: the three health regions from Alberta that submitted the first three highest number of specimens (Capital Health, Mistahia and Northern Lights), the rest of Alberta, and the out-of-province areas. To compare the diagnostic yield of NAT versus traditional viral diagnostic tests (EM and viral culture), all specimens that were tested by both NAT and one of the traditional assays were included in the analysis. Separate comparisons of the diagnostic yield using NAT versus traditional diagnostic methods were performed using the data from phase I and II of the study because different methods were used to extract the nucleic acid.

2.7 Exclusion for Seasonality analysis

For the analysis of monthly and seasonal distribution of the cases with enteric virus detected by NAT and/or EM, only one specimen from duplicate specimens with concordant NAT/EM results collected less than 15 days from the same patient were included in the analyses. If the duplicate specimens from a patient had discordant results by NAT and/or EM, both specimens were removed from the case-based analysis. After the removal of duplicate discordant or one of the duplicate concordant specimens, cases that were not tested by NAT were also excluded from the seasonality analysis.

Data on average Canadian climate (1971-2000) were downloaded from the Environmental Canada website.[70] Four sets of data: the number of days with rain ≥10 mm, the daily average temperature (in Celsius), the number of days with snow ≥0.2 cm and ≥5 cm respectively were plotted against the 12 months of a year in two separate scatter plots (Appendix D). Winter is designated to be from November to March because there are consistently more than five days in a month during those five months when there is >0.5 cm snow on the ground and when the average daily temperature is sub-zero. Summer is designated from June to August as there is only rain and no snow during those three months and the average temperature is always >12°C. April and May are designated as Spring and September and October as Autumn as those are the intervening months when the weather changes over.

2.8 Data Analysis

Significant difference in terms of categorical variables such as gender, health regions and type of submitters between: 1) included and excluded (not available for NAT) specimens in the comparison of NAT versus traditional methods (EM or culture), 2) included and excluded specimens to obtain cases for disease burden and seasonality analysis and, 3) cases tested positive for the four enteric viruses by NAT or EM and case tested negative by NAT and EM, were identified by Fisher's Exact Test or the χ^2 test as appropriate. Difference in age distribution for the above three types of comparison was identified by Mann Whitney-U test after the distribution of age was determined to be skewed by Shapiro-Wilk test and histogram.

While the detection of enteric virus by EM is dependent on the preservation of the morphology of the viral particles in the specimen, the detection by NAT is dependent on the stability and presence of nucleic acids. Because of the difference in the requirement for sample quality between the two detection methods, estimation for sensitivity and specificity was not made in this study in the absence of a gold standard. The comparison of diagnostic yield by NAT versus traditional viral diagnostic tests (EM and viral culture) was performed by both Kappa analysis and McNemar test. Kappa analysis was used to assess the agreement between the traditional methods and NAT, i.e., if the NAT is identifying the specific enteric viruses as detected by EM and viral culture respectively. The interpretation of Kappa value was as previously described: <0.20 = poor strength of agreement, 0-21-0.40 = fair, 0.41-0.60 = moderate,

0.61-0.80 = good, 0.81-1.00 = very good.[71] McNemar test was used to identify any significant difference in the detection of the enteric viruses by NAT versus EM, and NAT versus culture respectively.

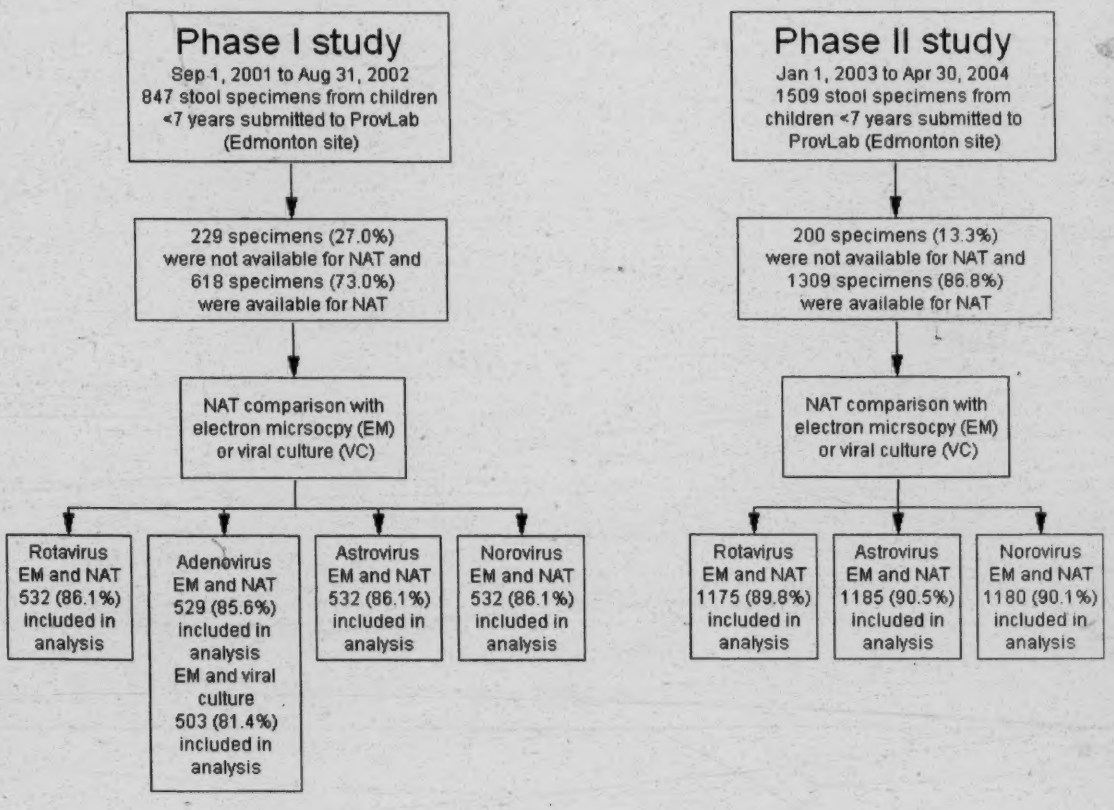
Age and gender of the patients, geographic regions, and types of medical practice of the submitting physicians were examined as potential factors associated with positive NAT and/or EM results for each enteric virus by multivariate binary logistic regression. For the seasonality analysis, the case of each enteric virus was classified into various seasons by the month of the case and the seasonal criteria described in section 2.7. The proportion of positive cases for each virus identified by NAT and/or EM in the corresponding season of the different years was combined and the differences among the four seasons for each virus were compared by χ^2 analysis. The level of significance was set at a p value of <0.05.

CHAPTER 3 NUCLEIC ACID AMPLIFICATION TEST AND TRADITIONAL VIRAL INVESTIGATIONS FOR ENTERIC VIRUS

3.1 Samples and study population

During phase I of the study, 847 stool specimens were submitted from children less than seven years of age to the Provincial Laboratory for Public Health (ProvLab) Edmonton site for viral studies and 618 specimens were available for nucleic acid amplification test (NAT) (Figure 3.1). Five hundred and thirty-two specimens were tested by electron microscope (EM) as well as by NAT for rotavirus, astrovirus and norovirus and those results were used in the comparison of the two methods of detection. For enteric adenovirus, 529 and 503 specimens were compared between NAT and EM, and NAT and viral culture respectively. During phase II, a total of 1,309 specimens were tested by NAT with 1,175 specimens specifically for rotavirus, 1,185 for astrovirus and 1,180 for norovirus and the respective specimens were included in the comparison of NAT versus EM for each of the virus.

Figure 3.1 Inclusion criteria for phase I and phase II of the study for the comparison of NAT with traditional viral studies (EM and viral culture)


Phase I study
Sep 1, 2001 to Aug 31, 2002
847 stool specimens from children <7 years submitted to ProvLab (Edmonton site)
229 specimens (27.0%) were not available for NAT and 618 specimens (73.0%) were available for NAT
NAT comparison with electron micrsocpy (EM) or viral culture (VC)
Rotavirus EM and NAT 532 (86.1%) included in analysis
Adenovirus EM and NAT 529 (85.6%) included in analysis
EM and viral culture 503 (81.4%) included in analysis
Astrovirus EM and NAT 532 (86.1%) included in analysis
Norovirus EM and NAT 532 (86.1%) included in analysis
Phase II study
Jan 1, 2003 to Apr 30, 2004
1509 stool specimens from children <7 years submitted to ProvLab (Edmonton site)
200 specimens (13.3%) were not available for NAT and 1309 specimens (86.8%) were available for NAT
NAT comparison with electron micrsocpy (EM) or viral culture (VC)
Rotavirus EM and NAT 1175 (89.8%) included in analysis
Astrovirus EM and NAT 1185 (90.5%) included in analysis
Norovirus EM and NAT 1180 (90.1%) included in analysis

3.2 Results of the traditional laboratory investigations

3.2.1 Electron microscopy

For the 2,356 specimens, EM was performed on 690 (81.5% of 847) and 1,344 (89.1% of 1,509) from phase I and phase II respectively. During phase I, 137 (20.0% of 690 specimens) tested positive by EM and in phase II, 317 (23.6% of 1,344 specimens) tested positive (p=0.06). There was significant difference in terms of the proportion of various enteric virus identified during phase I and phase II of the study with a higher proportion of rotavirus in phase II (Table 3.1).

Table 3.1 Results of electron microscopy from phase I and phase II of the study*‡

Electron microscopy (EM) results	No. of specimens in Phase I (% Total)	No. of specimens in Phase II (% Total)	Total
Negative by EM	553 (80.1%)	1,027 (76.4%)	1,580 (77.7%)
EM positive for adenovirus	28 (4.1%)	45 (3.3%)	73 (3.6%)
EM positive for rotavirus	91 (13.2%)	246 (18.3%)	337 (16.6%)
EM positive for SRSV†	18 (2.6%)	26 (1.9%)	44 (2.2%)
Total	690 (100.0%)	1,344 (100.0%)	2,034 (100.0%)

* In phase I, 157 stool specimens and in phase II, 165 specimens were not tested by EM

† SRSV - Small Round Structured Virus

‡ χ^2=9.62, df=3, p<0.05 for the comparison of the EM results between phase I and phase II

3.2.2 Viral culture

Viral culture was set up for 700 specimens (82.6% of 847) in phase I and 564 specimens (37.4% of 1,509) in phase II with the change in laboratory logarithm on July 21, 2003. During phase I, 30 of 700 specimens (4.3%) were positive for adenovirus by culture and 29 of 564 specimens (5.1%) were positive by culture in phase II (p=0.5).

3.2.3 Comparison of EM and viral culture for adenovirus

There were 992 specimens that were tested both by EM and viral culture for adenovirus (Table 3.2). The strength of agreement between EM and viral culture for adenovirus was low with a Kappa value of 0.23 demonstrating a significant discordance between viral culture and EM. Only 12 specimens were positive by both EM and culture.

3.2.4 Bacterial culture

Bacterial culture was set up for 165 specimens (19.5% of 847) in phase I and 275 specimens (18.2% of 1,509) in phase II. During phase I, seven of 165 specimens (4.2%) were positive for enteric bacteria including two for *Salmonella* species (sp.), two *Campylobacter* sp. and three *Aeromonas* sp. In phase II, another seven of 275 specimens (2.6%) were positive for enteric bacterial including three for *Salmonella* sp., one *Campylobacter* sp. and three for *Aeromonas* sp..

Table 3.2 Comparison of electron microscopy and viral culture for adenovirus from phase I and phase II of the study*†

	No. of specimens tested positive for adenovirus by EM (N=46)	No. of specimens tested negative for adenovirus by EM (N=946)	Total
No. of specimens tested positive for adenovirus by viral culture (N=46)	12 (26.1%)	34 (73.9%)	46
No. of specimens tested negative for adenovirus by viral culture (N=946)	34 (3.6%)	912 (96.4%)	946
Total	46 (4.6%)	946 (95.4%)	992

* Of the 2,356 specimens, 272 specimens were only set up for viral culture and had no EM, 1,042 specimens were only tested by EM and had no viral culture, and 50 specimens were not tested by EM and had no viral culture

† McNemar Test, p=1.0; Kappa value=0.23 for the comparison of EM and viral culture for adenovirus

3.3 Comparison of NAT versus EM/viral culture

3.3.1 Inclusion criteria: specimens available for NAT

Of those 847 specimens from phase I, 618 (73.0%) were available for NAT and from phase II, 1,309 (86.8%) of the 1,509 specimens were available. (χ^2=69.2, df=1, p<0.001). The distribution of the NAT-tested and not-tested specimens by month are shown in Figure 3.2. More than 60% of the submitted stool specimens per month in 24 of the 28 months of the study were available for NAT (except for 44.1% to 57.8% during September to December 2001) (Figure 3.3).

There was no significant difference in the median age of the patients from phase I (0.99 years, range: 0.00-6.97) and phase II of the study (1.01 years, range: 0.00-6.88). Combining the specimens from phase I and phase II, there was no significant difference between the median age of the patients whose specimens were tested by NAT (1.01 years) and the patients whose specimens were unavailable for NAT (0.93 years) (Table 3.3).

Twenty-six specimens were from patients with unknown gender and 22 of these specimens were available for NAT. Of the remaining 2,330 specimens, there was no significant difference between the proportion of specimens available for NAT from males and females respectively (Table 3.4). The male to female ratio for the NAT-tested specimens was 1.3:1.0.

Only 25 of the 2,356 specimens (1.1%) were from outside of Alberta (Table 3.5). Among the regions within Northern Alberta, there was significant difference in the

proportion of specimens that were available for and tested by NAT, ranging from 79.7% from Capital Health to 89.2% from Mistahia. The highest number of specimen tested by NAT was submitted from Capital Health (N=965) and the lowest number from Mistahia (N=199).

The types of clinical practice associated with 105 specimens were unknown. Of those 105 specimens, 99 were available for and tested by NAT and six were not tested by NAT. There was significant difference in the proportion of specimens available for NAT among the various type of clinical practice associated with the specimens (Table 3.6). Majority of specimens available for NAT were submitted for testing from a general pediatrician or a pediatric specialist or surgeon (N=1,265, 80.1%).

Figure 3.2 Monthly Distribution of number of specimens tested and not tested by NAT

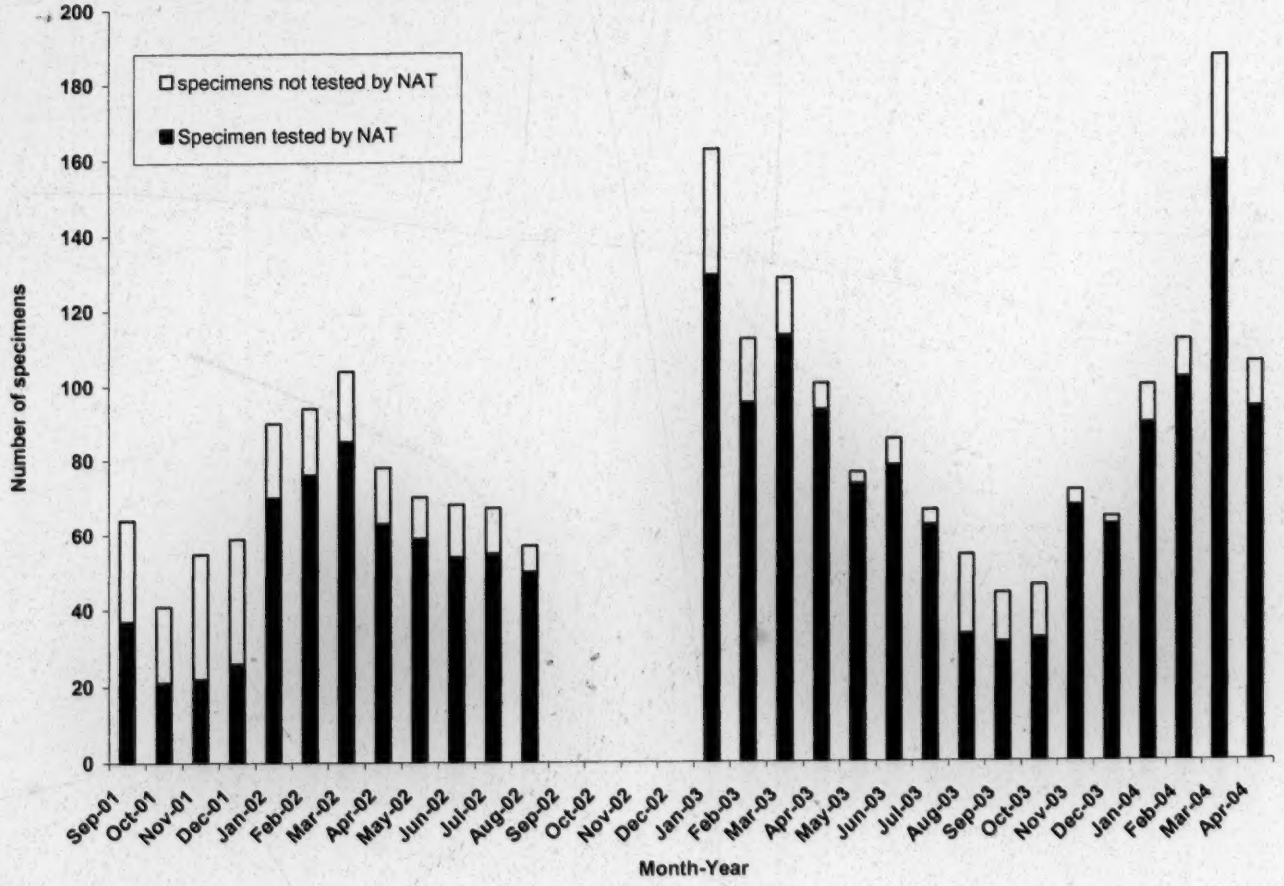


Figure 3.3 Proportion of specimens available for testing by NAT by month

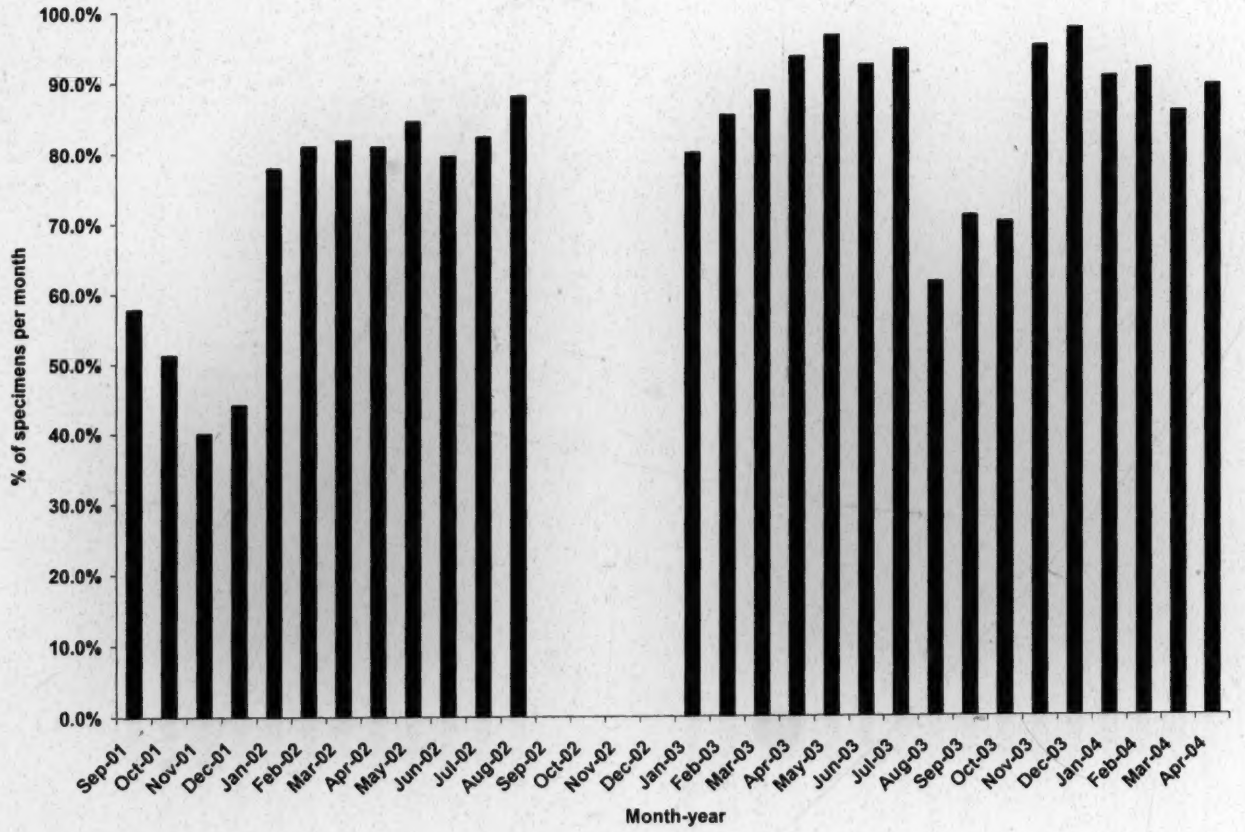


Table 3.3 Age distribution of specimens available and not available for testing by NAT

	Specimen tested by NAT	Specimens not tested by NAT
Mean age ± SD (years)	1.54±1.51	1.47±1.56
Median age (years)*	1.01	0.93
Minimum (age in years)	0.00	0.00
25th percentile (age in years)	0.43	0.35
75th percentile (age in years)	2.10	2.20
Maximum (age in years)	6.97	6.92

Age in categories	No. of specimen tested by NAT (% of total)	No. of specimen not tested by NAT (% of total)	Total
0 to <3 months	311 (78.3%)	86 (21.7%)	397
3 to <6 months	233 (80.9%)	55 (19.1%)	288
6 to <12 months	416 (83.9%)	80 (16.1%)	496
12 to <18 months	279 (81.6%)	63 (18.4%)	342
18 to <24 months	180 (82.9%)	37 (17.1%)	217
24 to <36 months	209 (83.3%)	42 (16.7%)	251
36 to <48 months	125 (83.3%)	25 (16.7%)	150
48 to <60 months	79 (78.2%)	22 (21.8%)	101
60 to <72 months	57 (90.5%)	6 (9.5%)	63
72 to <84 months	38 (74.5%)	13 (25.5%)	51
Total	1,927 (81.8%)	429 (18.2%)	2,356

*p=0.1, Mann-Whitney U test

Table 3.4 Gender distribution of specimens available and not available for testing by NAT*†

Gender	No. of specimen tested by NAT (% of total)	No. of specimen not tested by NAT (% of total)	Total
Male	1,079 (82.0%)	236 (18.0%)	1,315
Female	826 (81.4%)	189 (18.6%)	1,015
Total	1,905 (81.8%)	425 (18.2%)	2,330

* 26 specimens (22 available for NAT and four not available) from patients with unknown gender were excluded from the χ^2 analysis

† χ^2 test, df=1, p=0.7 for the comparison of the distribution between male and female

Table 3.5 Geographic distribution of specimens available and not available for testing by NAT*‡

Geographic regions	No. of specimen tested by NAT (% of total)	No. of specimen not tested by NAT (% of total)	Total
Capital Health (Region 10)	965 (79.7%)	246 (20.3%)	1,211
Mistahia (Region 13)	199 (89.2%)	24 (10.8%)	223
Northern Lights (Region 16)	337 (84.9%)	60 (15.1%)	397
Pooled regions†	363 (79.8%)	92 (20.2%)	455
Out of Province	62 (89.9%)	7 (10.1%)	25
Total	1,927 (81.8%)	429 (18.2%)	2,356

* One specimen (available for NAT) from unknown geographic region was excluded from the χ^2 analysis

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

‡ χ^2=15.4, df=3, $p<0.05$ for the comparison among regions after excluding out of province cases

Table 3.6 Distribution of type of submitters by specimens available and not available for testing by NAT*†

Type of Submitter	No. of specimen tested by NAT	No. of specimen not tested by NAT	Total
Pediatrician (general or specialist, or surgeons)	1,265 (80.1%)	315 (19.9%)	1,580
Family Physician or General Practitioner	546 (84.4%)	101 (15.6%)	647
Medical Officer of Health or Nurse-in-charge	17 (70.8%)	7 (29.2%)	24
Total	1,828 (81.8%)	423 (18.2%)	2,251

* 105 specimens (99 available for NAT and six not available for NAT) from submitters who were unclassified by the type of practice were excluded from the χ^2 analysis.

† χ^2=7.34, df=2, $p<0.05$ for the comparison of the distribution among the types of submitter

3.3.2 Comparison of traditional viral studies and NAT for rotavirus

The total number of stool specimens tested by NAT for rotavirus was 532 for phase I and 1,175 for phase II of the study. Of these, only the specimens that were tested by both EM and the TaqMan RT-PCR assay (N=1,707) were included in the comparison analysis.

3.3.2.1 NAT for Rotavirus

All 618 stool specimens from phase I were tested by the real time TaqMan RT-PCR assay for rotavirus and 123 (19.9%) were positive. During phase II, 1,298 of the 1,309 specimens were tested by NAT and 283 (21.6%) were positive. (p=0.3)

3.3.2.2 EM for Rotavirus

Electron microscopy was performed on 532 of the 618 (86.1%) specimens in phase I and 1,185 of the 1,309 (90.5%) specimens in phase II of the study. Seventy-nine specimens (14.9%) were tested as positive for rotavirus by EM in phase I and 232 (19.6%) were positive for rotavirus in phase II of the study. (χ^2=5.54, df=1, p<0.05)

3.3.2.3 Comparison of detection of rotavirus by EM and by NAT

The results of rotavirus as detected by EM versus NAT are tabulated in Table 3.7. There was significant increase in the detection of rotavirus by NAT, with a total of 111 specimens that had tested negative by EM for rotavirus tested positive for rotavirus by NAT in phase I (N=39, 7.3%) and phase II (N=72, 6.1%)

of the study. Thirteen specimens that had tested positive by EM were negative by NAT. Kappa values comparing EM and NAT for both phase I and phase II of the study were above 0.7 in phase I and phase II indicating excellent agreement between the two methods of detection. There is a higher proportion of samples tested positive by EM and negative by NAT in phase II of the study (12 of 208, 5.8%) as compared to phase I (1 of 78, 1.30%) (p=0.1).

Table 3.7 Comparison of detection of rotavirus by electron microscopy and nucleic acid amplification test*

Phase I: September 2001 to August 2002 (N=532)†

	No. of specimens tested positive for rotavirus by EM (N=79)	No. of specimens tested negative for rotavirus by EM (N=453)
No. of specimens tested positive for rotavirus by NAT (N=117)	78 (14.7%)	39 (7.3%)
No. of specimens tested negative for rotavirus by NAT (N=415)	1 (0.2%)	414 (77.8%)

Phase II: January 2003 to April 2004 (N=1,175)‡

	No. of specimens tested positive for rotavirus by EM (N=208)	No. of specimens tested negative for rotavirus by EM (N=967)
No. of specimens tested positive for rotavirus by NAT (N=268)	196 (16.7%)	72 (6.1%)
No. of specimens tested negative for rotavirus by NAT (N=907)	12 (1.0%)	895 (76.2%)

* In phase I, all 618 specimens were tested by NAT and 86 of the 618 specimens were not tested by EM. In phase II, 124 specimens were not tested by EM and 11 specimens were not tested for rotavirus by NAT.

† $p<0.001$, McNemar Test; Kappa value=0.75 for the comparison of EM and NAT

‡ $p<0.001$, McNemar Test; Kappa value=0.78 for the comparison of EM and NAT

3.3.3 Comparison of traditional viral studies and NAT for adenovirus

The LightCycler PCR assay for adenovirus was only performed on the specimens from phase I of the study. The total number of stool specimens tested by NAT for adenovirus was 615 (99.5% of 618) and of those, 529 specimens were tested by both NAT and EM and 503 by both NAT and viral culture.

3.3.3.1 NAT for adenovirus

Nucleic acid amplification for adenovirus was performed only in the phase I study. Twenty-seven (4.39%) of the 615 NAT-tested specimens were positive for adenovirus.

3.3.3.2 Electron microscopy and viral culture for adenovirus

Electron microscopy was performed on 532 (86.1%) and viral culture for 505 (81.7%) of the 618 specimens. Twenty-three (4.3%) specimens were positive for adenovirus by EM and adenovirus was isolated by viral culture in 21 (4.2%) specimens.

3.3.3.3 Comparison of detection of adenovirus by EM, viral culture and NAT

The results of viral culture, EM and NAT are shown in Table 3.8. There was no significant difference in the identification of adenovirus by NAT as compared with EM and viral culture. The concordance between EM and NAT was excellent with a Kappa value of 0.96. Only seven specimens tested positive by both NAT and viral culture for adenovirus and five of those specimens also

tested positive by EM and two were not tested by EM. The Kappa value comparing viral culture and NAT was 0.34 indicating a poor concordance.

Table 3.8 Comparison of detection of adenovirus by electron microscopy and adenovirus serotypes 40 and 41 by nucleic acid amplification test (NAT) and detection of adenovirus by viral culture and adenovirus serotypes 40 and 41 by NAT*

Phase I: September 2001 to August 2002 (N=529)†

	No. of specimens tested positive for adenovirus by EM (N=23)	No. of specimens tested negative for adenovirus by EM (N=506)
No. of specimens tested positive for adenovirus by NAT	23 (4.3%)	2 (0.4%)
No. of specimens tested negative for adenovirus by NAT	0 (0.0%)	504 (95.3%)

Phase I: September 2001 to August 2002 (N=503)‡

	No. of specimens tested positive for adenovirus by viral culture (N=21)	No. of specimens tested negative for adenovirus by viral culture (N=482)
No. of specimens tested positive for adenovirus by NAT	7 (1.4%)	10 (2.0%)
No. of specimens tested negative for adenovirus by NAT	14 (2.8%)	472 (93.8%)

* Adenovirus NAT was only performed in phase I with 615 of the 618 specimens tested by NAT. Eighty-six specimens were not tested by both EM and NAT, and 113 were not tested by both viral culture and NAT.

† p=0.5, McNemar Test; Kappa value=0.96 for the comparison of EM and NAT

‡ p=0.5, McNemar Test; Kappa value=0.34 for the comparison of viral culture and NAT

3.3.4 Comparison of traditional viral studies and NAT for astrovirus

The total number of stool specimens tested by NAT for astrovirus was 532 for phase I and 1,168 for phase II of the study. Of these specimens, 1,700 were tested by both NAT and EM and they were included in the comparison analysis.

3.3.4.1 NAT for astrovirus

During phase I of the study, all 618 stool specimens were tested for astrovirus by conventional RT-PCR and 21 (3.4%) were positive. For phase II, 1,291 of the 1,309 (98.6%) specimens were tested and 43 (3.3%) specimens were positive. ($p=0.9$, χ^2 test, $df=1$)

3.3.4.2 EM for astrovirus (as SRSV)

Astrovirus was identified only as SRSV by EM. Electron microscopy was performed on 532 of the 618 (86.1%) specimens and 1,185 of the 1,309 (90.5%) specimens for the two study periods. Fourteen (1.9%) specimens were identified as having SRSV by EM in phase I and 18 (1.0%) in phase II. ($p=0.1$, χ^2 test, $df=1$)

3.3.4.3 Comparison of detection of astrovirus by EM and by NAT

The results of SRSV as detected by EM versus astrovirus as detected by NAT are tabulated in Table 3.9. For both phase I and phase II of the study, >50% of the specimens positive for SRSV by EM tested positive as astrovirus by NAT, 71.4% in phase I and 66.7% in phase II, respectively. In phase I of the study, there was no significant difference in the identification of astrovirus comparing EM and NAT and the Kappa value was 0.61 indicating good

agreement between EM and NAT. For phase II of the study, there was significant increase in the detection of astrovirus as compared to EM with a Kappa value of 0.4, which is still indicative of fair agreement between the two detection methods.

Table 3.9 Comparison of detection of astrovirus by electron microscopy and nucleic acid amplification test*

Phase I: September 2001 to August 2002 (N=532)†

	No. of specimens tested positive for SRSV by EM (N=14)	No. of specimens tested negative for SRSV by EM (N=518)
No. of specimens tested positive for astrovirus by NAT (N=18)	10 (1.9%)	8 (1.5%)
No. of specimens tested negative for astrovirus by NAT (N=514)	4 (0.8%)	510 (95.9%)

Phase II: January 2003 to April 2004 (N=1,168)‡

	No. of specimens tested positive for SRSV by EM (N=18)	No. of specimens tested negative for SRSV by EM (N=1,150)
No. of specimens tested positive for astrovirus by NAT (N=40)	12 (1.0%)	28 (2.4%)
No. of specimens tested negative for astrovirus by NAT (N=1,128)	6 (0.5%)	1,122 (96.1%)

* In phase I, all 618 specimens were tested by NAT and 86 of the 618 specimens were not tested by EM. In phase II, 124 specimens were not tested by EM and 18 specimens were not tested for astrovirus by NAT.

† p=0.4, McNemar Test; Kappa value=0.61 for the comparison of EM and NAT

‡ p<0.001, McNemar Test; Kappa value=0.40 for the comparison of EM and NAT

3.3.5 Comparison of traditional viral studies and NAT for norovirus

The total number of stool specimens tested by NAT for norovirus was 532 for phase I and 1,180 for phase II of the study. Of these specimens, 1,712 specimens were tested by both NAT and EM and were included in the comparison analysis.

3.3.5.1 NAT for norovirus

During phase I, all 618 stool specimens were tested and 40 specimens (6.5%) were positive for norovirus by both conventional RT-PCR and real-time LightCycler RT-PCR. During phase II, 1,304 of the 1,309 specimens that were submitted (99.6%) specimens were tested by the TaqMan RT-PCR and 169 of the 1,304 specimens (13.0%) were positive for norovirus.

3.3.5.2 EM for norovirus (as SRSV)

Similar to astrovirus, norovirus was identified as SRSV by EM. The EM findings for SRSV during phase I and phase II of the study of have been described under the heading "EM for astrovirus (as SRSV)" in section 3.3.4.

3.3.5.3 Comparison of detection of norovirus by EM and by NAT

The results of astrovirus as detected by EM versus NAT are tabulated in Table 3.10. As compared to astrovirus, a smaller proportion of the specimens that had tested positive for SRSV by EM tested positive by NAT for norovirus, with only two specimens (14.3%) in phase I and two in phase II (11.1%). There was significant difference in the number of specimens tested positive for

norovirus as compared to the detection of SRSV by EM for phase I and II of the study and the Kappa values for both phases of the study were <0.1 indicating poor agreement between NAT and EM.

Table 3.10 Comparison of detection of norovirus by electron microscopy and nucleic acid amplification test*

Phase I: September 2001 to August 2002 (N=532)†

	No. of specimens tested positive for SRSV by EM (N=14)	No. of specimens tested negative for SRSV by EM (N=518)
No. of specimens tested positive for norovirus by NAT (N=37)	2 (0.4%)	35 (6.6%)
No. of specimens tested negative for norovirus by NAT (N=495)	12 (2.3%)	483 (90.8%)

Phase II: January 2003 to April 2004 (N=1,180)‡

	No. of specimens tested positive for SRSV by EM (N=18)	No. of specimens tested negative for SRSV by EM (N=1,162)
No. of specimens tested positive for norovirus by NAT (N=157)	2 (0.2%)	155 (13.1%)
No. of specimens tested negative for norovirus by NAT (N=1,023)	16 (1.4%)	1,007 (85.3%)

* In phase I, all 618 specimens were tested by NAT and 86 of the 618 specimens were not tested by EM. In phase II, 124 specimens were not tested by EM and five specimens were not tested for norovirus by NAT

† $p<0.05$, McNemar Test; Kappa value=0.04 for the comparison of EM and NAT

‡ $p<0.001$, McNemar Test; Kappa value= -0.005 for the comparison of EM and NAT

3.3.6 Comparison of traditional viral studies and NAT for astrovirus and norovirus

Since both astrovirus and norovirus were identified as SRSV by EM, comparison of EM with combined NAT results for these two viruses were also performed (Table 3.11). A total of 532 and 1,167 specimens in phase I and phase II respectively that were tested by EM as well as by NAT for both astrovirus and norovirus were included in the analysis. By combining the NAT results, there was significant difference in the number of specimens tested positive for norovirus or astrovirus as compared to the detection of SRSV by EM for both phase I and phase II. The Kappa value for phase I was 0.3 indicating fair agreement, and the Kappa value in phase II was 0.1 indicating poor agreement, which is likely related to the high number of norovirus positive specimens in phase II.

Table 3.11 Comparison of detection of astrovirus and norovirus by electron microscopy and nucleic acid amplification test*

Phase I: September 2001 to August 2002 (N=532)†

	No. of specimens tested positive for SRSV by EM (N=14)	No. of specimens tested negative for SRSV by EM (N=518)
No. of specimens tested positive for astrovirus or norovirus by NAT (N=53)	11 (2.1%)	42 (7.9%)
No. of specimens tested negative for astrovirus and norovirus by NAT (N=479)	3 (0.6%)	476 (89.5%)

Phase II: January 2003 to April 2004 (N=1,167)‡

	No. of specimens tested positive for SRSV by EM (N=18)	No. of specimens tested negative for SRSV by EM (N=1,149)
No. of specimens tested positive for astrovirus or norovirus by NAT (N=193)	14 (1.2%)	179 (15.3%)
No. of specimens tested negative for astrovirus and norovirus by NAT by NAT (N=974)	4 (0.3%)	970 (83.1%)

* In phase I, all 618 specimens were tested by NAT for both astrovirus and norovirus and 86 of the 618 specimens were not tested by EM. In phase II, 1290 specimens were by NAT for both astrovirus and norovirus and 123 specimens were not tested for by EM

† $p<0.001$, McNemar Test; Kappa value=0.3 for the comparison of EM and NAT

‡ $p<0.001$, McNemar Test; Kappa value=0.1 for the comparison of EM and NAT

CHAPTER 4 EPIDEMIOLOGY OF ENTERIC VIRUS IN NORTHERN ALBERTA

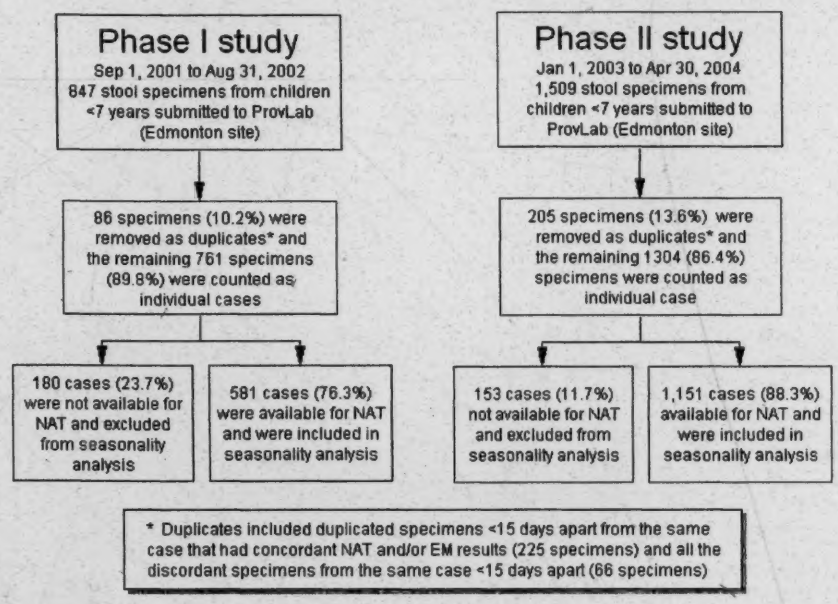
4.1 Samples and study population

For the seasonality analysis, 86 specimens (10.2%) were removed from phase I of the study and 205 (13.6%) specimens were removed from phase II of the study (Figure 4.1) because they were:

- duplicated specimens <15 days apart from the same case that had concordant NAT and/or EM results (225 specimens), or
- all the discordant specimens from the same case <15 days apart (66 specimens).

Of the 291 excluded specimens, 66 were specimens from 30 individual cases with discordant results.

Figure 4.1 Inclusion criteria for phase I and phase II of the study for the comparison of NAT with traditional viral studies (EM and viral culture)


Phase I study
Sep 1, 2001 to Aug 31, 2002
847 stool specimens from children <7 years submitted to ProvLab (Edmonton site)
86 specimens (10.2%) were removed as duplicates* and the remaining 761 specimens (89.8%) were counted as individual cases
180 cases (23.7%) were not available for NAT and excluded from seasonality analysis
581 cases (76.3%) were available for NAT and were included in seasonality analysis
Phase II study
Jan 1, 2003 to Apr 30, 2004
1,509 stool specimens from children <7 years submitted to ProvLab (Edmonton site)
205 specimens (13.6%) were removed as duplicates* and the remaining 1304 (86.4%) specimens were counted as individual case
153 cases (11.7%) not available for NAT and excluded from seasonality analysis
1,151 cases (88.3%) available for NAT and were included in seasonality analysis
* Duplicates included duplicated specimens <15 days apart from the same case that had concordant NAT and/or EM results (225 specimens) and all the discordant specimens from the same case <15 days apart (66 specimens)

After removing the 291 duplicate specimens, 333 cases were excluded from the seasonality analysis because they were not available for NAT (Figure 4.1). After application of the inclusion criteria, a total of 1,732 cases, 581 (76.3% of 761) cases from phase I and 1,151 (88.3% of 1,304) cases from phase II of the study were included in the seasonality analysis (χ^2=50.5, df=1, p<0.001). The characteristics of 1,732 cases included in the seasonality analysis were compared to the characteristics of the 363 individual cases that were excluded because:

- they were either not available for NAT, or
- because the NAT and/or EM results were discordant for the individual case

Among the 2,095 cases, there was no significant difference between the median age of phase I (1.00 year) and phase II of the study (1.04 year) (p=0.6, Mann-Whitney U test). Combining the cases from phase I and phase II, there was no significant difference in the median age of the cases that were included in the seasonality analysis (1.01 years) and the median age of the excluded cases (0.93 years) (Table 4.1).

Twenty-six cases had unknown gender. The proportion of male in phase I (54.2%) and phase II (55.6%) of the study were similar (p=0.7) and combining the cases from phase I and phase II, the proportions of cases included in the seasonality analysis among male and female were not significantly different (Table 4.2). The male to female ratio of the included cases was 1.3:1.0.

Sixty-six of the 2,094 cases (3.2%) were from outside of Alberta and the proportion of cases included in the seasonality analysis from each region was different, ranging from 80.2% of the cases from Capital Health to 90.6% from Mistahia (Table 4.3). From within Northern Alberta, the highest number of cases included in the seasonality analysis was submitted from Capital Health (N=844, 48.7%) and the lowest number from Mistahia (N=194, 11.2%). For phase I of the study, the proportion of included cases among different regions in Northern Alberta was significantly different with Mistahia > rest of Northern Alberta > Northern Lights > Capital Health (Table 4.4). In phase II, the proportion of cases from Mistahia > Northern Lights > Capital Health > rest of Northern Alberta but the proportional difference in terms of inclusion and exclusion in the seasonality analysis was not significant.

The types of clinical practice associated with 103 cases (4.9%) were unknown, and the proportion of cases included in the seasonality analysis for the various types of practice was summarized in Table 4.5. The majority of cases included in the seasonality analysis was submitted by a general pediatrician or a pediatric specialist or surgeon (N=1,090, 62.9%), followed by family physician or general practitioner (N=531, 30.7%). For both phase I and phase II of the study, the proportion of included case was: family physician or general practitioner > pediatric specialist or sub-specialist > medical officer of Health or nurse-in-charge but only in phase I that the difference in distribution was significant (Table 4.6).

After excluding the cases from out-of province, the cases with unknown region/submitter type/gender, and the few cases submitted by Medical Officers of

Health or nurse-in-charge, the only significant difference between included and excluded cases was the higher proportion of inclusion in phase II as compared to phase I of the study, as there was no significant difference among geographic regions or types of clinical practice in terms of inclusion and exclusion by multivariate analysis (Table 4.7).

Table 4.1 Age distribution of individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis

	Individual case included in seasonality analysis	Individual case excluded in seasonality analysis
Mean age ± SD (years)	1.57 ±1.54	1.49 ±1.56
Median age (years)*	1.03	0.91
Minimum (age in years)	0.00	0.00
25th percentile (age in years)	0.45	0.35
75th percentile (age in years)	2.13	2.08
Maximum (age in years)	6.97	6.92

Age in categories	No. of individual case included in seasonality analysis (N=1,732)	No. of individual case excluded in seasonality analysis (N=363)	Total
0 to <3 months	276 (79.1%)	73 (20.9%)	349
3 to <6 months	198 (81.5%)	45 (18.5%)	243
6 to <12 months	366 (83.6%)	72 (16.4%)	438
12 to <18 months	254 (83.8%)	49 (16.2%)	303
18 to <24 months	167 (85.6%)	28 (14.4%)	195
24 to <36 months	194 (84.3%)	36 (15.7%)	230
36 to <48 months	111 (82.2%)	24 (17.8%)	135
48 to <60 months	75 (78.1%)	21 (21.9%)	96
60 to <72 months	54 (91.5%)	5 (8.5%)	59
72 to <84 months	37 (78.7%)	10 (21.3%)	47
Total	1,732 (82.7%)	363 (17.3%)	2,095

* p=0.08, Mann-Whitney U test

Table 4.2 Distribution of gender of individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis*†

Gender	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Male	968 (83.9%)	186 (16.1%)	1,154
Female	742 (81.1%)	173 (18.9%)	915
Total	1,710 (82.6%)	359 (17.4%)	2,069

* 26 cases (4 excluded and 22 included in the seasonality analysis) from patients with unknown gender were excluded from the χ^2 analysis

† χ^2 test, df=1, p=0.1, for the comparison of the distribution between males and females

Table 4.3 Geographic distribution of individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis*

Geographic regions	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Capital Health (Region 10)	844 (80.2%)	208 (19.8%)	1,052
Mistahia (Region 13)	194 (90.6%)	20 (9.4%)	241
Northern Lights (Region 16)	315 (84.9%)	56 (15.1%)	371
Pooled regions†	320 (81.8%)	71 (18.2%)	391
Out of Province	58 (87.9%)	8 (12.1%)	66
Total	1,731 (82.7%)	363 (17.3%)	2,094

* One specimen from unknown geographic region was not included in the table

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

Table 4.4 Geographic distribution within Northern Alberta of individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis in phase I & phase II of the study*

Phase I: September 2001 to August 2002‡

Geographic regions	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Capital Health (Region 10)	264 (70.0%)	113 (30.0%)	377
Mistahia (Region 13)	57 (87.7%)	8 (12.3%)	65
Northern Lights (Region 16)	85 (78.7%)	23 (21.3%)	108
Pooled regions†	144 (80.0%)	36 (20.0%)	180
Total	550 (75.3%)	180 (24.7%)	730

Phase II: January 2003 to April 2004§

Geographic regions	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Capital Health (Region 10)	580 (85.9%)	95 (14.1%)	675
Mistahia (Region 13)	137 (91.9%)	12 (8.1%)	149
Northern Lights (Region 16)	230 (87.5%)	33 (12.5%)	263
Pooled regions†	176 (83.4%)	35 (16.6%)	211
Total	1,123 (86.5%)	175 (13.5%)	1,298

* A total of 67 cases were excluded from the table and χ^2 analysis: 39 cases from phase 1 of the study including one case from unknown geographic region (included in the seasonality analysis) and 38 cases from out of province (30 included and eight excluded in the seasonality analysis) and 28 out-of-province cases in phase II (all included in the seasonality analysis)

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

‡ χ^2=13.8, df=3, p<0.001, for the comparison among regions after excluding out of province cases in phase I of the study

§ χ^2 test, df=3, p=0.1, for the comparison among regions after excluding out of province cases in phase II of the study

Table 4.5 Distribution of type of submitters by individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis

Type of Submitter	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Pediatrician (general or specialist, or surgeons)	1,090 (80.7%)	260 (19.3%)	1,350
Family Physician or General Practitioner	531 (85.8%)	88 (14.2%)	619
Medical Officer of Health or Nurse-in-charge	16 (69.6%)	7 (30.4%)	23
Unclassified by the type of practice	95 (92.2%)	8 (7.8%)	103
Total	1,732 (82.7%)	363 (17.3%)	2,095

Table 4.6 Distribution of type of submitters by individual cases included and excluded (excluded because of no NAT or discordant results) in the seasonality analysis in phase I & phase II of the study*

Phase I: September 2001 to August 2002†

Type of Submitter	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Pediatrician (general or specialist, or surgeons)	328 (71.0%)	134 (29.0%)	462
Family Physician or General Practitioner	198 (80.8%)	47 (19.2%)	245
Medical Officer of Health or Nurse-in-charge	6 (66.7%)	3 (33.3%)	9
Total	532 (74.3%)	184 (25.7%)	716

Phase II: January 2003 to April 2004‡

Type of Submitter	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Total
Pediatrician (general or specialist, or surgeons)	762 (85.8%)	126 (14.2%)	888
Family Physician or General Practitioner	333 (89.0%)	41 (11.0%)	374
Medical Officer of Health or Nurse-in-charge	10 (71.4%)	4 (28.6%)	14
Total	1,105 (86.6%)	171 (13.4%)	1,276

* 103 cases (95 included in the seasonality analysis and eight excluded from the seasonality analysis) from submitters unclassified by the type of practice were excluded from the table and χ^2 analysis

† χ^2=8.4, df=2, p<0.05 for the comparison of the distribution among the types of submitter in phase I of the study

‡ χ^2 test, df=2, p=0.08, for the comparison of the distribution among the types of submitter in phase II of the study

Table 4.7 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters by individual cases in terms of inclusion and exclusion in seasonality analysis (excluded because of no NAT or discordant results)*

	No. of individual case included in seasonality analysis (% of total)	No. of individual case excluded in seasonality analysis (% of total)	Odds ratio (95% CI)
Phase I and Phase II of study (‡p<0.001)			
Phase I	515 (75.5%)	167 (24.5%)	-
Phase II‡	1,093 (88.4%)	144 (11.6%)	2.4 (1.9 - 3.1)
Age in categories (p=0.2)			
0 to <3 months	253 (79.1%)	67 (20.9%)	-
3 to <6 months	190 (84.1%)	36 (15.9%)	1.3 (0.8 - 2.1)
6 to <12 months	344 (86.2%)	55 (13.8%)	1.5 (1.0 - 2.2)
12 to <18 months	233 (83.8%)	45 (16.2%)	1.2 (0.8 - 1.8)
18 to <24 months	154 (87.0%)	23 (13.0%)	1.6 (1.0 - 2.8)
24 to <36 months	173 (85.2%)	30 (14.8%)	1.3 (0.8 - 2.1)
36 to <48 months	101 (82.8%)	21 (17.2%)	1.2 (0.7 - 2.1)
48 to <60 months	72 (77.4%)	21 (22.6%)	0.8 (0.5 - 1.5)
60 to <72 months	53 (94.6%)	3 (5.4%)	4.5 (1.3 - 14.9)
72 to <84 months	35 (77.8%)	10 (22.2%)	1.0 (0.5 - 2.1)
Gender (p=0.1)			
Male	914 (85.2%)	159 (14.8%)	-
Female	694 (82.0%)	152 (18.0%)	0.8 (0.6 - 1.0)
Regions within Alberta (p=0.3)			
Capital Health (Region 10)	836 (81.9%)	185 (18.1%)	
Mistahia (Region 13)	176 (90.3%)	19 (9.7%)	1.5 (0.9 - 2.8)
Northern Lights (Region 16)	305 (85.9%)	50 (14.1%)	1.2 (0.9 - 1.7)
Pooled regions†	291 (83.6%)	57 (16.4%)	1.0 (0.7 - 1.5)
Type of submitter (p=0.2)			
Pediatrician (general or specialist, or surgeons)	1,106 (82.6%)	233 (17.4%)	-
Family Physician or General Practitioner	502 (86.6%)	78 (13.4%)	1.3 (0.9 - 1.8)
Total	1,608 (83.8%)	311 (16.2%)	1,919

* A total of 176 cases (24 excluded and 152 included in seasonality analysis) were excluded from the multivariate binary logistic regression analysis: a) from out of province (N=66), b) from unknown region (N=1), c) with an unknown type of submitters (N=74), d) submitted by either Medical Officers of Health or public health (N=15) and cases with unknown gender (N=20)

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

4.2 Descriptive epidemiology of cases with identifiable enteric virus

For phase I of the study, 187 cases (32.2%) were positive by NAT and/or EM for one or more of the enteric viruses, and for phase II, 440 cases (38.2%) were positive by NAT and/or EM (χ^2=6.1, df=1, p<0.05). The monthly distribution of cases with positive and negative NAT and/or EM result is shown in Figure 4.2. Comparing the same month of the different years, there were more cases included in the seasonality analysis in each month of the year in phase II as compared to phase I except for August 2002 (phase I, N=49) and August 2003 (phase II, N=31) as well as September 2001 (phase I, N=37) and September 2003 (phase II, N=30), respectively. Of the 28 months during phase I and phase II of the study, seven months had <20% of the cases tested positive by NAT and/or EM, 10 months had 20 to <40% of cases tested positive, and 11 months had 40 to <60% of cases tested positive (Figure 4.3).

The median age of cases with positive results by NAT and/or EM for enteric virus (1.10 years, range: 0.01-6.77) was significantly higher than the age of cases with negative results (0.98 years, range: 0.00-6.97) (Table 4.8). The odds of having a positive result by NAT and/or EM for age three months to <72 months were significantly higher than the odds for cases younger than three months. The lower limit of the 95% confidence interval for the odds ratios of the respective age group was >5.0 for the age ranging from six months to 18 months. Excluding the 22 cases whose gender was unknown, there was no significant difference in terms of the gender of cases with positive and negative NAT and/or

EM results respectively (Table 4.9). The male to female ratio of the cases with positive NAT and/or EM results was 1.3:1.0.

Fifty-eight of the 1,732 cases were from outside of Alberta and the proportion of cases tested positive for enteric virus was different among the different regions, ranging from 27.6% of the cases from out-of-province to 53.6% from Mistahia (Table 4.10). From within Northern Alberta, the highest number of specimens tested positive by NAT and/or EM was submitted from Capital Health (N=260) and the lowest number from Mistahia (N=104). The difference in geographic distribution of positive and negative cases within Alberta was significant for both phase I and phase II of the study (Table 4.11).

The types of clinical practice associated with 95 cases were unknown and the proportion of cases tested positive by NAT and/or EM among the various types of clinical practice are summarized in Table 4.12. Excluding cases from unknown type of submitter, the proportion of cases tested positive by NAT and EM submitted by a family physician or general practitioner was significantly higher than other types of practice for both phase I and phase II of the study (Table 4.13). The highest number of positive cases had specimens submitted by a general pediatrician or a pediatric sub-specialist or surgeon (N=346, 58.0%), followed by the cases with specimens submitted by a family physician or general practitioner (N=243, 40.7%).

Excluding the cases with unknown type of submitters, the cases from unknown region or out-of-province, and the few cases submitted by either Medical Officers of Health or nurse-in-charge, there is significant difference in the

proportion of the two types of submitters within different regions of Alberta (Table 4.14). Using multivariate binary logistic regression after excluding the cases from out-of province, the cases with unknown region/submitter type/gender, and the few cases submitted by Medical Officers of Health or nurse-in-charge, significant factors associated with the identification of one or more enteric virus in the stool samples by NAT and/or EM were: age from three months to <60 months, Mistahia as a region in Alberta, and family physician or general practitioner as submitters (Table 4.15). Stool samples collected from children < three months and >60 months old were not significantly associated with the identification of enteric virus.

Figure 4.2 Monthly Distribution of the cases tested positive by NAT and/or electron microscopy for enteric viruses including rotavirus, adenovirus, astrovirus, norovirus, and small round structured virus and negative cases

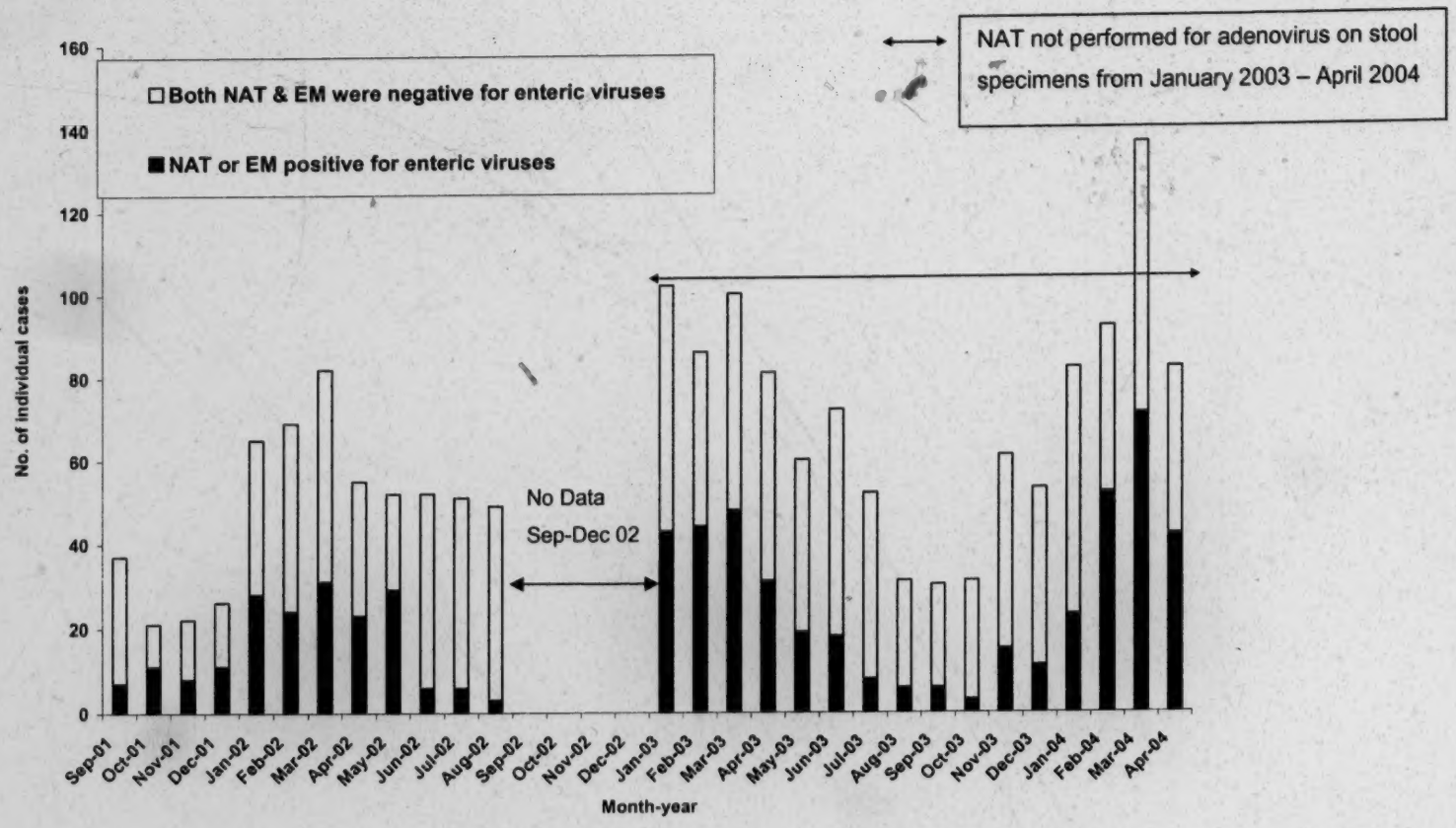


Figure 4.3 Monthly Distribution of proportion of cases tested positive by NAT and/or electron microscopy for enteric viruses including rotavirus, adenovirus, astrovirus, norovirus and small round structured virus

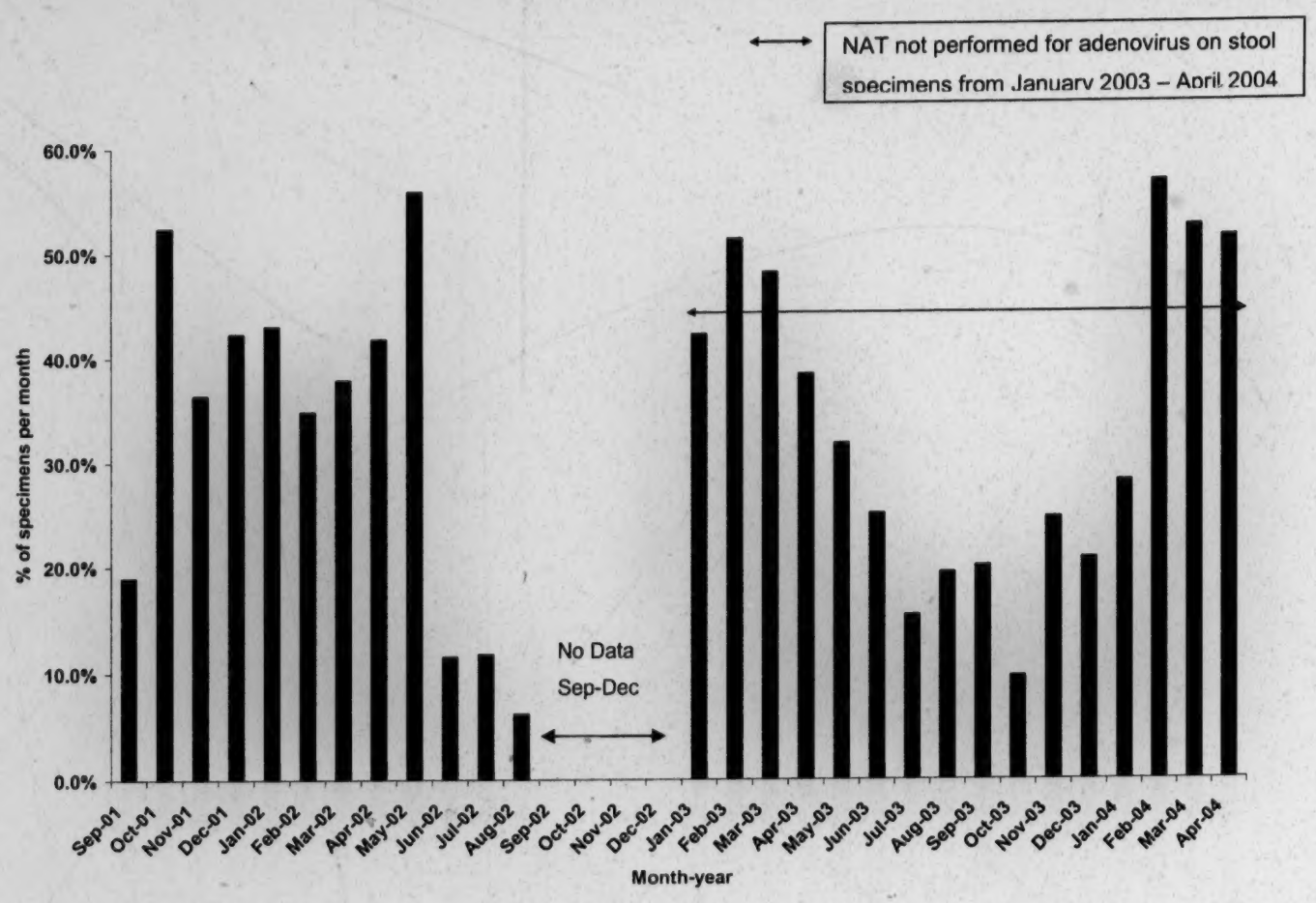


Table 4.8 Age distribution of individual cases tested positive and negative for enteric viruses including rotavirus, enteric adenovirus, astrovirus, norovirus and small round structured virus by NAT and/or EM*

	No. of cases tested positive for enteric viruses by NAT and/or EM (N=627)	No. of cases tested negative for enteric viruses (N=1,105)
Mean age ± SD (years)	1.47±1.23	1.63±1.69
Median age (years)	1.10	0.98
Minimum (age in years)	0.01	0.00
25th percentile (age in years)	0.70	0.32
75th percentile (age in years)	1.85	2.43
Maximum (age in years)	6.77	6.97

Age in categories	No. of cases tested positive for enteric viruses by NAT and/or EM (% of total)	No. of cases tested negative for enteric viruses (% of total)	Odds ratio
0 to <3 months	31 (11.2%)	245 (88.8%)	-
3 to <6 months†	74 (37.4%)	124 (62.6%)	4.7 (2.9- 7.5)
6 to <12 months†	175 (47.8%)	191 (52.2%)	7.2 (4.7-11.1)
12 to <18 months†	137 (53.9%)	117 (46.1%)	9.2 (5.9-14.4)
18 to <24 months†	74 (44.3%)	93 (55.7%)	6.3 (3.9-10.2)
24 to <36 months†	72 (37.1%)	122 (62.9%)	4.7 (2.9- 7.5)
36 to <48 months†	26 (23.4%)	85 (76.6%)	2.4 (1.4- 4.3)
48 to <60 months†	19 (25.3%)	56 (74.7%)	2.6 (1.4- 5.0)
60 to <72 months†	12 (22.2%)	42 (77.8%)	2.3 (1.1- 4.7)
72 to <84 months	7 (18.9%)	30 (81.1%)	1.9 (0.8- 4.6)
Total	627 (36.2%)	1,105 (63.8%)	-

* NAT not performed for adenovirus on stool specimens from January 2003 – April 2004

† Comparison of positive and negative NAT and/or EM among different age groups using the 0 to <3 months as the reference age group by binary logistic regression, df=9, Wald=137.8, $p<0.05$

Table 4.9 Gender distribution of the cases tested positive and negative for enteric viruses including rotavirus, enteric adenovirus, astrovirus, norovirus and small round structured virus by NAT and/or EM*†‡

Gender	No. of cases tested positive for enteric viruses by NAT and/or EM (% of total)	No. of cases tested negative for enteric viruses (% of total)	Total
Male	348 (36.0%)	620 (64.0%)	968
Female	269 (36.3%)	473 (63.7%)	742
Total	617 (36.1%)	1,093 (63.9%)	1,710

* 22 cases (12 positive and 10 negative for enteric virus by NAT and/or EM) from patients with unknown gender were excluded from the table and χ^2 analysis.

† NAT not performed for adenovirus on stool specimens from January 2003 – April 2004

‡ χ^2 test, df=1, p=0.9, for the comparison of the distribution among male and female

Table 4.10 Geographic distribution of individual cases tested positive for enteric viruses including rotavirus, enteric adenovirus, astrovirus and norovirus by NAT and/or EM and negative cases*†

Geographic regions	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Capital Health (Region 10)	260 (30.8%)	584 (69.2%)	844
Mistahia (Region 13)	104 (53.6%)	90 (46.4%)	194
Northern Lights (Region 16)	122 (38.7%)	193 (61.3%)	315
Pooled regions‡	125 (39.1%)	195 (60.9%)	320
Out of Province	16 (27.6%)	42 (72.4%)	58
Total	627 (36.2%)	1,105 (63.8%)	1,731

* One case from unknown geographic region and tested negative for enteric virus was excluded from the table.

† NAT not performed for adenovirus on stool specimens from January 2003 – April 2004

‡ David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

Table 4.11 Geographic distribution within Northern Alberta of individual cases tested positive for enteric viruses including rotavirus, enteric adenovirus, astrovirus and norovirus by NAT and/or EM and negative cases in phase I & phase II of the study*

Phase I: September 2001 to August 2002‡

Geographic regions	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Capital Health (Region 10)	66 (25.0%)	198 (75.0%)	264
Mistahia (Region 13)	29 (50.9%)	28 (49.1%)	57
Northern Lights (Region 16)	30 (35.3%)	55 (64.7%)	85
Pooled regions†	56 (38.9%)	88 (61.1%)	144
Total	181 (32.9%)	369 (67.1%)	550

Phase II: January 2003 to April 2004§

Geographic regions	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Capital Health (Region 10)	194 (33.4%)	386 (66.6%)	580
Mistahia (Region 13)	75 (54.7%)	62 (45.3%)	137
Northern Lights (Region 16)	92 (40.0%)	138 (60.0%)	230
Pooled regions†	69 (39.2%)	107 (60.8%)	176
Total	430 (38.3%)	693 (61.7%)	1,123

* A total of 59 cases: one case from unknown geographic region (tested negative for enteric virus) and 30 cases from out of province (6 positive and 24 negative for enteric virus) from phase I of the study, and 28 cases from out of province (10 positive and 18 negative for enteric virus) from phase II of the study were excluded from the table and χ^2 analysis

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

‡ χ^2=18.4, df=3, p<0.001, for the comparison among regions after excluding out of province cases in phase I of the study

§ χ^2=21.8, df=3, p<0.001, for the comparison among regions after excluding out of province cases in phase II of the study

Table 4.12 Distribution of type of submitters of individual cases tested positive for enteric viruses including rotavirus, enteric adenovirus, astrovirus and norovirus by NAT and/or EM and negative cases*

Type of Submitter	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Pediatrician (general or specialist, or surgeons)	346 (31.7%)	744 (68.3%)	1,090
Family Physician or General Practitioner	243 (45.8%)	288 (54.2%)	531
Medical Officer of Health or Nurse-in-charge	8 (50.0%)	8 (50.0%)	16
Unclassified by the type of practice	30 (31.6%)	65 (68.4%)	95
Total	627 (36.2%)	1,105 (63.8%)	1,732

* NAT not performed for adenovirus on stool specimens from January 2003 – April 2004

Table 4.13 Distribution of type of submitters individual cases tested positive for enteric viruses including rotavirus, enteric adenovirus, astrovirus and norovirus by NAT and/or EM and negative cases in phase I & phase II of the study*

Phase I: September 2001 to August 2002†

Type of Submitter	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Pediatrician (general or specialist, or surgeons)	83 (25.3%)	245 (74.7%)	328
Family Physician or General Practitioner	86 (43.4%)	112 (56.6%)	198
Medical Officer of Health or Nurse-in-charge	1 (16.7%)	5 (83.3%)	6
Total	170 (32.0%)	362 (68.0%)	532

Phase II: January 2003 to April 2004‡

Type of Submitter	No. of cases tested positive by NAT and/or EM (% of total)	No. of cases tested negative by NAT and/or EM (% of total)	Total
Pediatrician (general or specialist, or surgeons)	263 (34.5%)	499 (65.5%)	762
Family Physician or General Practitioner	157 (47.1%)	176 (52.9%)	333
Medical Officer of Health or Nurse-in-charge	7 (70.0%)	3 (30.0%)	10
Total	427 (38.6%)	678 (61.4%)	1,105

* A total of 95 cases: 49 cases from submitters unclassified by the type of practice (17 positive and 32 negative for enteric viruses) from phase I, and 46 cases from submitters unclassified by the type of practice (13 positive and 33 negative for enteric viruses) from phase I were excluded from the table and χ^2 analysis

† χ^2=19.3, df=2, p<0.001, for the comparison of the distribution among the types of submitter in phase I of the study

‡ χ^2=19.8, df=2, p<0.001, for the comparison of the distribution among the types of submitter in phase II of the study

Table 4.14 Distribution of two types of submitters for cases included for seasonality analysis within Alberta*‡

Geographic regions	Type of submitters		Total
	Pediatrician (general or specialist, including surgeons)	Family Physician or General Practitioner	
Capital Health (Region 10)	757 (92.0%)	66 (8.0%)	823
Mistahia (Region 13)	26 (14.9%)	149 (85.1%)	175
Northern Lights (Region 16)	229 (75.3%)	75 (24.7%)	304
Pooled regions†	75 (25.4%)	220 (74.6%)	295
Total	1,087 (68.1%)	510 (31.9%)	1,597

* A total of 135 cases: one case from unknown region, 58 out-of-province cases, 68 cases with unknown type of submitters and eight cases submitted by either Medical Officers of Health or public health were excluded from the χ^2 analysis

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

‡ χ^2=698.6, df=3, p<0.001 for the comparison of the two types of submitters, pediatrician (general or specialist, including surgeons) and family physician or general practitioner, within different regions in Alberta

Table 4.15 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters for cases tested positive for enteric viruses by NAT and/or EM*

	No. of cases tested positive for enteric viruses (% of total)	No. of cases tested negative for enteric viruses (% of total)	Odds ratio (95% CI)
Phase I and Phase II of study (p=0.06)			
Phase I	164 (32.2%)	345 (67.8%)	-
Phase II	411 (38.4%)	660 (61.6%)	1.3 (1.0 - 1.6)
Age in categories (‡p<0.001)			
0 to <3 months	27 (10.8%)	222 (89.2%)	-
3 to <6 months‡	70 (38.0%)	114 (62.0%)	5.0 (3.0 - 8.2)
6 to <12 months‡	163 (48.9%)	170 (51.1%)	7.1 (4.5 - 11.2)
12 to <18 months‡	124 (53.4%)	108 (46.6%)	8.0 (4.9 - 12.9)
18 to <24 months‡	69 (45.1%)	84 (54.9%)	5.9 (3.5 - 10.0)
24 to <36 months‡	63 (36.8%)	108 (63.2%)	4.1 (2.5 - 6.9)
36 to <48 months‡	24 (24.2%)	75 (75.8%)	2.2 (1.2 - 4.2)
48 to <60 months‡	19 (26.4%)	53 (73.6%)	2.4 (1.2 - 4.7)
60 to <72 months	10 (19.2%)	42 (80.8%)	1.6 (0.7 - 3.7)
72 to <84 months	6 (17.1%)	29 (82.9%)	1.5 (0.6 - 3.9)
Gender (p=0.5)			
Male	322 (35.7%)	579 (64.3%)	-
Female	253 (37.3%)	426 (62.7%)	1.1 (0.9 - 1.3)
Regions within Alberta (§p<0.05)			
Capital Health (Region 10)	250 (30.6%)	567 (69.4%)	-
Mistahia (Region 13)§	96 (54.9%)	79 (45.1%)	1.8 (1.2 - 2.8)
Northern Lights (Region 16)	118 (39.2%)	183 (60.8%)	1.2 (0.9 - 1.7)
Pooled regions†	111 (38.7%)	176 (61.3%)	1.0 (0.7 - 1.4)
Type of submitter (IIp<0.05)			
Pediatrician (general or specialist, or surgeons)	340 (31.5%)	739 (68.5%)	-
Family Physician or General PractitionerII	235 (46.9%)	266 (53.1%)	1.5 (1.1 - 2.1)
Total	575 (36.4%)	1,005 (63.6%)	1,580

* A total of 152 cases (52 positive and 100 negative for enteric virus) were excluded from the multivariate binary logistic regression: a) from out of province (N=58), b) from unknown region (N=1), c) with unknown type of submitters

(N=68), d) submitted by either Medical Officers of Health or public health (N=8) and cases with unknown gender (N=17)

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

4.3 Frequency of each enteric virus

4.3.1 Single and mixed infections

The proportion of cases tested negative by NAT and proportion of cases tested positive for rotavirus, adenovirus, astrovirus, norovirus and SRSV for phase I and phase II study respectively are shown in Figure 4.4. Combining the two studies with NAT not performed for adenovirus in phase II, of the 1,732 cases, rotavirus was the most commonly identified virus (N=385), followed by 166 cases of norovirus, 53 of adenovirus, 61 of astrovirus and six cases of SRSV. For the 627 individual cases that were tested positive for the one or more enteric virus, 586 cases had only one enteric virus identified and 41 cases had mixed infections. Rotavirus with norovirus was the most common type of mixed infections (23 cases), followed by seven cases of rotavirus with astrovirus, four cases of norovirus with adenovirus, three cases of norovirus with astrovirus, two cases of rotavirus with astrovirus and norovirus, and one case of rotavirus with adenovirus and one case of rotavirus with astrovirus and norovirus (Figure 4.5).

Figure 4.4 Distribution of the enteric viruses identified by nucleic acid amplification (NAT) and/or electron microscopy (EM) represented as percentage of total # of cases for Phase I and Phase II study. (Duplicate specimens as defined by <15 days apart or discordant paired samples removed before analysis)

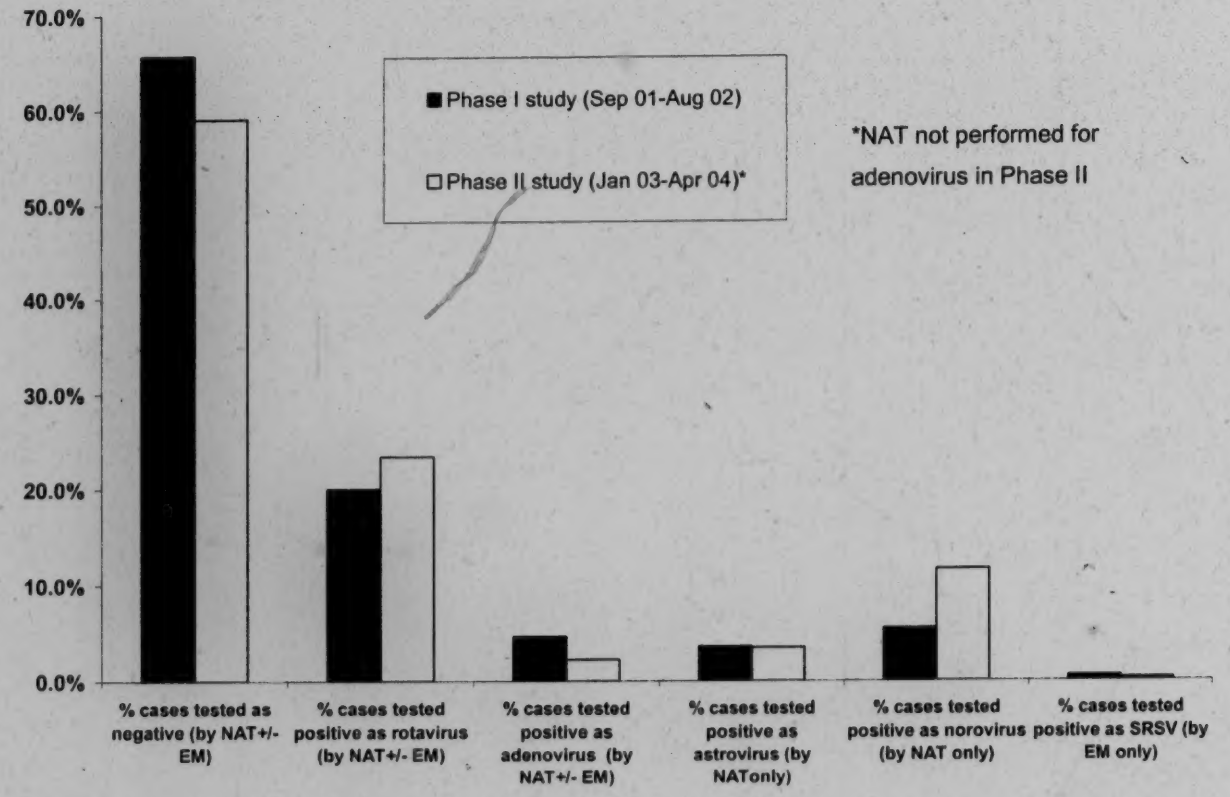
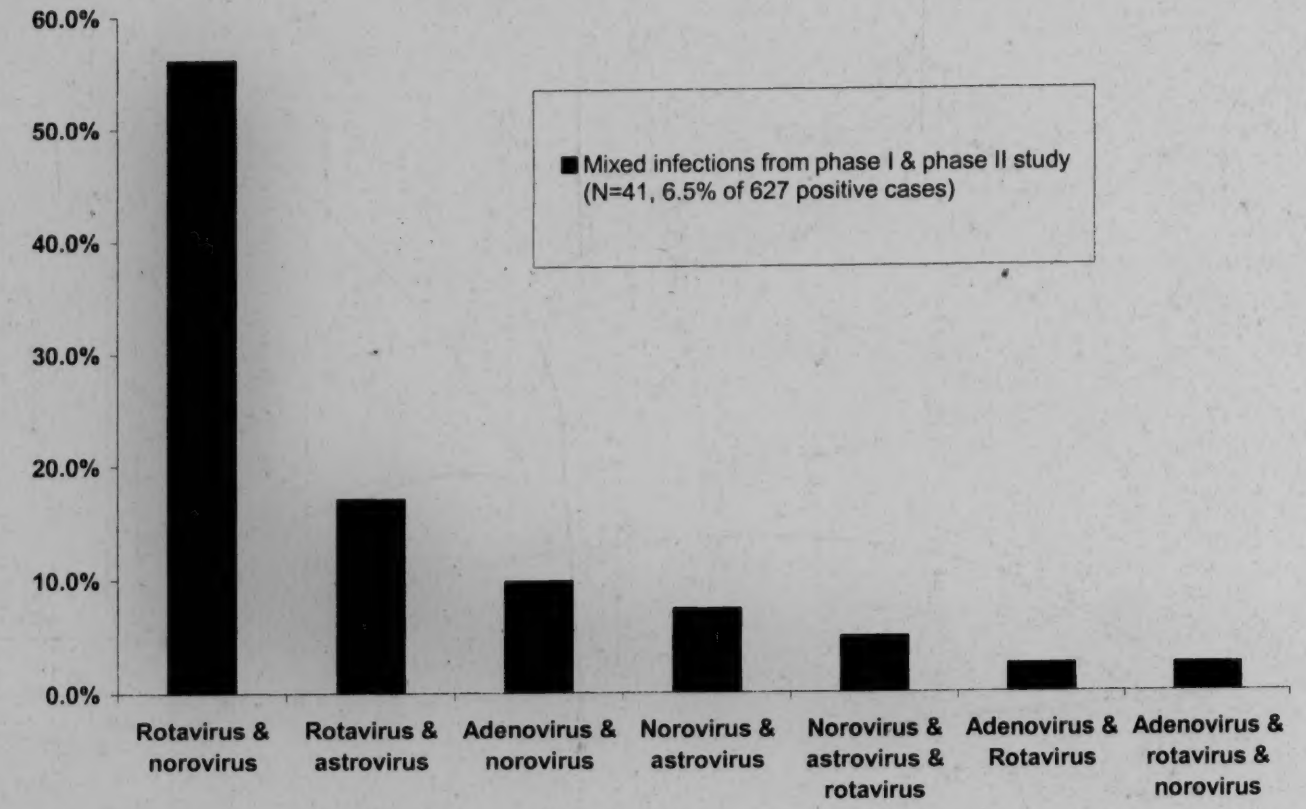

70.0%
60.0%
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
Phase I study (Sep 01-Aug 02)
Phase II study (Jan 03-Apr 04)*
*NAT not performed for adenovirus in Phase II
% cases tested as negative (by NAT+/- EM)
% cases tested positive as rotavirus (by NAT+/- EM)
% cases tested positive as adenovirus (by NAT+/- EM)
% cases tested positive as astrovirus (by NATonly)
% cases tested positive as norovirus (by NAT only)
% cases tested positive as SRSV (by EM only)

Figure 4.5 Distribution of the different type of mixed infections identified by nucleic acid amplification (NAT) and/or electron microscopy (EM) (NAT not performed for adenovirus on stool specimens from January 2003 – April 2004)



4.3.2 Rotavirus

There was no significant difference in terms of the proportion of positive case between phase I (20%) and phase II (23%) of the study using combined positive results by EM and NAT for rotavirus (p=0.1). The factors that were significantly associated with a positive NAT and/or EM for rotavirus were age <36 months, age between 48 months to 60 months, as well as cases from Mistahia (Table 4.16). There is significant difference in the median age of cases within Alberta with median age in Mistahia > pooled regions > Northern Lights > Capital Health (Table 4.17).

4.3.3 Enteric adenovirus

For enteric adenovirus, cases from Northern Lights Region were significantly associated with a positive NAT and/or EM result while age was not a significant factor (Table 4.18).

4.3.4 Astrovirus

The proportion of positive case for astrovirus for phase I (3.6%) and phase II (3.5%) of the study was essentially the same and no significant associated factor was identified for a positive NAT for astrovirus (Table 4.19).

4.3.5 Norovirus

There was a significantly higher proportion of positive cases of norovirus in phase II (11.7%) as compared to phase I (5.5%) of the study (χ^2=16.9, df=1, p<0.001). Phase II of the study, age <48 months and gender as male were

significant factors associated with a positive NAT for norovirus with multivariate binary logistic regression (Table 4.20).

Table 4.16 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters for cases tested positive for rotavirus by NAT and/or EM*

	No. of cases tested positive for rotavirus (% of total)	No. of cases tested negative for rotavirus (% of total)	Odds ratio (95% CI)
Phase I and Phase II of study (p=0.3)			
Phase I	103 (20.2%)	406 (79.8%)	-
Phase II	255 (24.0%)	806 (76.0%)	1.2 (0.9 - 1.5)
Age in categories (‡p<0.001)			
0 to <3 months	15 (6.0%)	233 (94.0%)	-
3 to <6 months‡	35 (19.2%)	147 (80.8%)	3.6 (1.9 - 6.8)
6 to <12 months‡	111 (33.7%)	218 (66.3%)	7.2 (4.1 - 12.8)
12 to <18 months‡	87 (37.7%)	144 (62.3%)	8.3 (4.6 - 15.0)
18 to <24 months‡	46 (30.3%)	106 (69.7%)	6.2 (3.3 - 11.7)
24 to <36 months‡	32 (18.7%)	139 (81.3%)	3.2 (1.6 - 6.1)
36 to <48 months‡	13 (13.3%)	85 (86.7%)	2.0 (0.9 - 4.5)
48 to <60 months‡	14 (19.4%)	58 (80.6%)	3.2 (1.4 - 7.1)
60 to <72 months	3 (5.8%)	49 (94.2%)	0.8 (0.2 - 2.8)
72 to <84 months	2 (5.7%)	33 (94.3%)	0.8 (0.2 - 3.8)
Gender (p=0.4)			
Male	210 (23.5%)	683 (76.5%)	-
Female	148 (21.9%)	529 (78.1%)	0.9 (0.7 - 1.1)
Regions within Alberta (§p<0.001)			
Capital Health (Region 10)	156 (19.2%)	657 (80.8%)	-
Mistahia (Region 13)§	73 (42.0%)	101 (58.0%)	2.5 (1.6 - 3.9)
Northern Lights (Region 16)	68 (22.7%)	231 (77.3%)	1.1 (0.8 - 1.5)
Pooled regions†	61 (21.5%)	223 (78.5%)	0.9 (0.6 - 1.4)
Type of submitter (p=0.4)			
Pediatrician (general or specialist, or surgeons)	214 (19.9%)	859 (80.1%)	-
Family Physician or General Practitioner	144 (29.0%)	353 (71.0%)	1.1 (0.8 - 1.6)
Total	358 (22.8%)	1,212 (77.2%)	1,570

* A total of 162 cases (23 cases tested positive for rotavirus) were excluded from the multivariate binary logistic regression: a) from out of province (N=58), b) from unknown region (N=1), c) with unknown type of submitters (N=68), d) submitted by either Medical Officers of Health or public health (N=8), e) cases with unknown gender (N=17) and f) 10 cases not tested by NAT for rotavirus

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

Table 4.17 Age distribution within Northern Alberta of all cases included in the seasonality analysis*‡

	Capital Health (Region 10) (N=844)	Mistahia (Region 13) (N=194)	Northern Lights (Region 16) (N=315)	Pooled regions† (N=320)
Mean age ± SD (years)	1.44±1.51	1.95±1.62	1.47±1.42	1.79±1.61
Median age (years)	0.85	1.35	1.00	1.31
Minimum (age in years)	0.00	0.02	0.01	0.01
25th percentile (age in years)	0.33	0.83	0.49	0.70
75th percentile (age in years)	2.01	2.60	1.91	2.26
Maximum (age in years)	6.94	6.41	6.90	6.97

* A total of 59 cases, one case from unknown geographic region and phase I of the study, 30 cases from out of province in phase I and 28 cases from out of province in phase II were excluded the table Kruskal Wallis analysis

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

‡ Kruskal Wallis Test, df=3, $p<0.001$, for the comparison among regions after excluding out of province cases

Table 4.18 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters for cases tested positive for adenovirus by NAT and/or EM*

	No. of cases tested positive for adenovirus (% of total)	No. of cases tested negative for adenovirus (% of total)	Odd ratio (95% CI)
Age in categories (p=0.7)			
0 to <3 months	2 (2.3%)	245 (97.7%)	-
3 to <6 months	2 (3.2%)	179 (96.8%)	1.8 (0.2 - 14.0)
6 to <12 months	5 (5.2%)	312 (94.8%)	2.3 (0.4 - 12.6)
12 to <18 months	3 (4.6%)	214 (95.4%)	1.9 (0.3 - 12.9)
18 to <24 months	7 (12.7%)	147 (87.3%)	6.2 (1.1 - 33.4)
24 to <36 months	2 (4.2%)	161 (95.8%)	2.0 (0.3 - 15.9)
36 to <48 months	0 (0.0%)	95 (100.0%)	Not applicable
48 to <60 months	2 (9.1%)	72 (90.9%)	3.3 (0.4 - 25.9)
60 to <72 months	1 (5.3%)	51 (94.7%)	1.9 (0.1 - 24.4)
72 to <84 months	0 (0.0%)	33 (100.0%)	Not applicable
Gender (p=0.1)			
Male	11 (3.8%)	279 (96.2%)	-
Female	13 (6.0%)	203 (94.0%)	1.9 (0.8 - 4.5)
Regions within Alberta (‡p<0.05)			
Capital Health (Region 10)	5 (2.0%)	249 (98.0%)	-
Mistahia (Region 13)	5 (10.0%)	45 (90.0%)	3.0 (0.6 - 15.6)
Northern Lights (Region 16)‡	9 (11.4%)	70 (88.6%)	5.8 (1.8 - 19.3)
Pooled regions†	5 (4.1%)	118 (95.9%)	1.1 (0.2 - 5.2)
Type of submitter (p=0.2)			
Pediatrician (general or specialist, or surgeons)	10 (3.1%)	313 (96.9%)	-
Family Physician or General Practitioner	14 (7.7%)	169 (92.3%)	2.1 (0.6 - 6.9)
Total	24 (4.7%)	482 (95.3%)	506

* Phase II where no NAT was performed for enteric adenovirus was excluded from the table and analysis. A total of 75 cases (3 cases tested positive for adenovirus) were also excluded from the multivariate binary logistic regression: a) from out of province (N=30), b) from unknown region (N=1), c) with unknown

type of submitters (N=32), d) submitted by either Medical Officers of Health or public health (N=3), e) cases with unknown gender (N=6) and f) three cases not tested by NAT for adenovirus

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

Table 4.19 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters for cases tested positive for astrovirus by NAT and/or EM*

	No. of cases tested positive for astrovirus (% of total)	No. of cases tested negative for astrovirus (% of total)	Odd ratio (95% CI)
Phase I and Phase II of study (p=0.7)			
Phase I	19 (3.7%)	490 (96.3%)	-
Phase II	35 (3.3%)	1,019 (96.7%)	0.9 (0.5 - 1.6)
Age in categories (p=0.3)			
0 to <3 months	2 (0.8%)	245 (99.2%)	-
3 to <6 months	3 (1.6%)	179 (98.4%)	1.9 (0.3 - 11.8)
6 to <12 months	13 (4.0%)	312 (96.0%)	4.7 (1.0 - 21.2)
12 to <18 months	15 (6.6%)	214 (93.4%)	7.5 (1.7 - 33.5)
18 to <24 months	6 (3.9%)	147 (96.1%)	4.2 (0.8 - 21.5)
24 to <36 months	9 (5.3%)	161 (94.7%)	6.1 (1.3 - 28.7)
36 to <48 months	3 (3.1%)	95 (96.9%)	3.5 (0.6 - 21.4)
48 to <60 months	0 (0.0%)	72 (100.0%)	-
60 to <72 months	1 (1.9%)	51 (98.1%)	2.2 (0.2 - 25.3)
72 to <84 months	2 (5.7%)	33 (94.3%)	6.1 (0.8 - 45.5)
Gender (p=0.9)			
Male	31 (3.5%)	861 (96.5%)	-
Female	23 (3.4%)	648 (96.6%)	1.0 (0.6 - 1.7)
Regions within Alberta (p=0.4)			
Capital Health (Region 10)	21 (2.6%)	788 (97.4%)	-
Mistahia (Region 13)	5 (2.9%)	168 (97.1%)	0.7 (0.2 - 2.1)
Northern Lights (Region 16)	11 (3.7%)	287 (96.3%)	1.3 (0.6 - 2.7)
Pooled regions†	17 (6.0%)	266 (94.0%)	1.5 (0.7 - 3.5)
Type of submitter (p=0.3)			
Pediatrician (general or specialist, or surgeons)	39 (2.7%)	1,039 (97.3%)	-
Family Physician or General Practitioner	25 (5.1%)	470 (94.9%)	1.5 (0.7 - 3.0)
Total	54 (3.5%)	1,509 (96.5%)	1,563

* A total of 169 cases (7 cases tested positive for astrovirus) were excluded from the multivariate binary logistic regression: a) from out of province (N=58), b) from unknown region (N=1), c) with unknown type of submitters (N=68), d) submitted by either Medical Officers of Health or public health (N=8), e) cases with unknown gender (N=17) and f) 17 cases not tested by NAT for astrovirus

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

Table 4.20 Multivariate binary logistic regression of age, gender, regions within Alberta, and types of submitters for cases tested positive for norovirus by NAT and/or EM*

	No. of cases tested positive for norovirus (% of total)	No. of cases tested negative for norovirus (% of total)	Odd ratio (95% CI)
Phase I and Phase II of study (‡p<0.05)			
Phase I	28 (5.5%)	481 (94.5%)	-
Phase II‡	116 (10.9%)	951 (89.1%)	2.1 (1.4 - 3.2)
Age in categories (§p<0.05)			
0 to <3 months	8 (3.2%)	241 (96.8%)	-
3 to <6 months§	26 (14.1%)	158 (85.9%)	5.1 (2.2 - 11.5)
6 to <12 months§	39 (11.8%)	291 (88.2%)	3.9 (1.8 - 8.6)
12 to <18 months§	21 (9.1%)	210 (90.9%)	2.8 (1.2 - 6.5)
18 to <24 months	11 (7.2%)	142 (92.8%)	2.3 (0.9 - 5.8)
24 to <36 months§	17 (9.9%)	154 (90.1%)	3.1 (1.3 - 7.5)
36 to <48 months§	11 (11.1%)	88 (88.9%)	3.8 (1.5 - 9.8)
48 to <60 months	4 (5.6%)	68 (94.4%)	1.7 (0.5 - 5.8)
60 to <72 months	4 (7.7%)	48 (92.3%)	2.6 (0.7 - 8.9)
72 to <84 months	3 (8.6%)	32 (91.4%)	3.1 (0.8 - 12.4)
Gender (‖p<0.05)			
Male	71 (7.9%)	827 (92.1%)	-
Female‖	73 (10.8%)	605 (89.2%)	1.4 (1.0 - 2.0)
Regions within Alberta (p=0.6)			
Capital Health (Region 10)	73 (9.0%)	741 (91.0%)	-
Mistahia (Region 13)	13 (7.5%)	161 (92.5%)	0.6 (0.3 - 1.3)
Northern Lights (Region 16)	31 (10.3%)	270 (89.7%)	1.0 (0.7 - 1.6)
Pooled regions†	27 (9.4%)	260 (90.6%)	0.9 (0.5 - 1.6)
Type of submitter (p=0.2)			
Pediatrician (general or specialist, or surgeons)	95 (8.8%)	981 (91.2%)	-
Family Physician or General Practitioner	49 (9.8%)	461 (90.2%)	1.3 (0.8 - 2.2)
Total	144 (9.1%)	1,432 (90.9%)	1,576

* A total of 156 cases (23 cases tested positive for norovirus) were excluded from the multivariate binary logistic regression: a) from out of province (N=58), b) from unknown region (N=1), c) with unknown type of submitters (N=68), d) submitted by either Medical Officers of Health or public health (N=8), e) cases with unknown gender (N=17) and f) four cases not tested by NAT for norovirus

† David Thompson, East Central, Westview, Crossroad, Aspen, Lakeland, Peace, Keeweetinok Lakes, and Northwestern Health Region (Region 6, 7, 8, 9, 11, 12, 14, 15, 17)

4.4 Seasonality of the four enteric viruses

A total of ten seasonal periods were included in the seasonality analysis: Autumn 2001, Winter 2001-02, Spring 2002, Summer 2002, Winter 2002-03 (3 months only), Spring 2003, Summer 2003, Autumn 2003, Winter 2003-04 and Spring 2004 (1 month only). There were only six cases of SRSV (0.3% of the 1,732 cases) identified by EM that were not tested as either astrovirus or norovirus by NAT and seasonality analysis was not performed for SRSV. Seasonality analysis for rotavirus and enteric adenovirus was based on all the positive cases identified by NAT and/or EM for these two viruses. For astrovirus and norovirus, only cases specifically tested positive by NAT for the respective viruses were included in the seasonality analysis. The monthly distribution of the number and the proportion of the cases tested positive for each of the four enteric viruses are shown from Figure 4.6 to Figure 4.9.

4.4.1 Seasonality of rotavirus

There were a total of 385 cases of rotavirus detected and there were four months in the 28-month study period when there was no rotavirus identified: August 2002, August 2003, September 2003, and November 2003 (Appendix E). The monthly proportion of cases tested positive for rotavirus is shown in Figure 4.6. March and April were the months with the highest proportion of positive rotavirus cases. There was significant difference in the seasonal distribution of rotavirus with the higher proportion of positive cases in the Spring and Winter (Table 4.21).

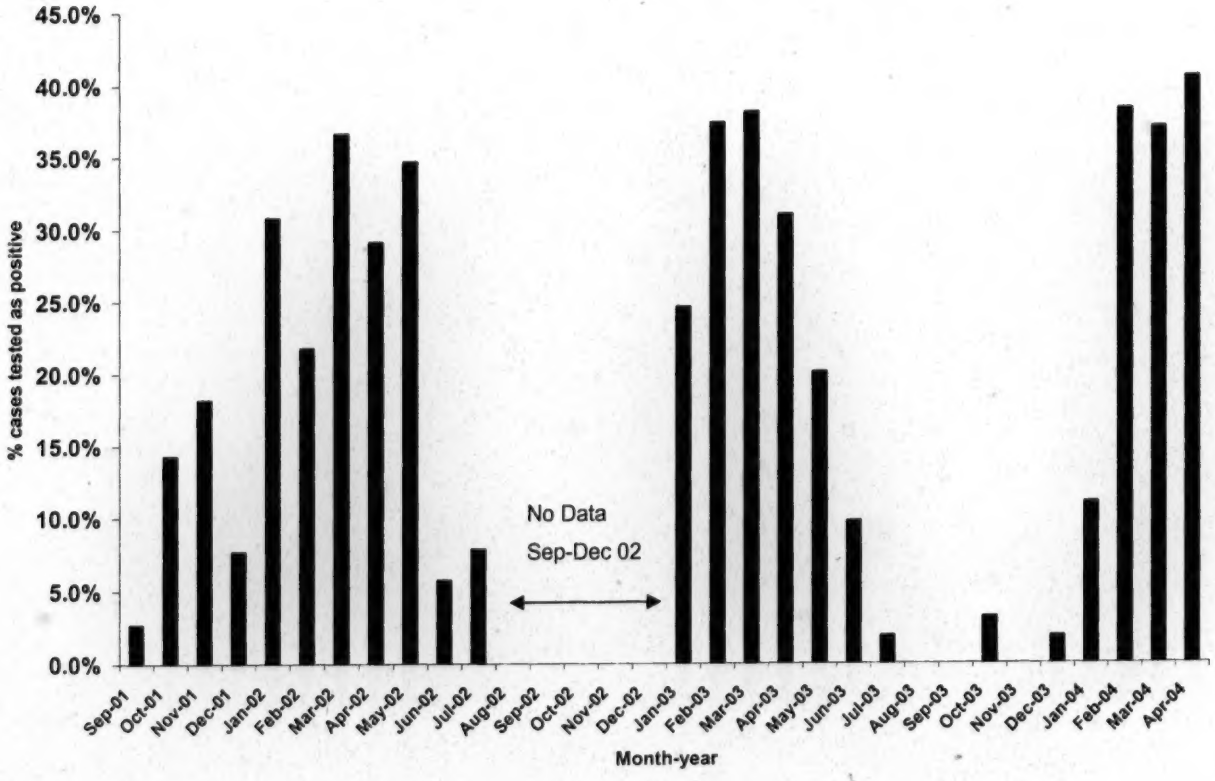
Table 4.21 Seasonal distribution of cases tested positive and negative for rotavirus by nucleic acid amplification test (NAT) and/or electron microscopy (EM)*†

Season	No. of cases tested positive for rotavirus by NAT and/or EM (%)	No. of cases tested negative for rotavirus by NAT and/or EM (%)	Total
Autumn	5 (4.2%)	114 (95.8%)	119
Winter	261 (26.7%)	715 (73.3%)	976
Spring	104 (31.5%)	226 (68.5%)	330
Summer	15 (4.9%)	292 (95.1%)	307
Total	385 (22.2%)	1,347 (77.8%)	1,732

* Over the 28 month of the phase I and phase II of the study: Autumn encompassed a 4-month period, Winter, a 13-month period, Spring, a 5-month period and Summer, a 6-month period

† χ^2=103.7, df=3, p<0.001, for the comparison of the distribution among the different seasons

Figure 4.6 Monthly distribution of the proportion of cases tested positive by nucleic acid amplification test and/or electron microscopy for rotavirus (N=385)



4.4.2 Seasonality of enteric adenovirus

There were 53 cases of adenovirus detected and there were four months in the 28-month study period when there was no enteric adenovirus identified: January 2002, September 2003, November 2003, and March 2004 (Appendix F). The monthly proportion of cases tested positive for enteric adenovirus is shown in Figure 4.7. Except for a high positive rate of 23.8% for adenovirus in October 2001, the remaining 23 months during the phase I and phase II of the study had a positive rates of <8.0% per month for enteric adenovirus. There was significant difference in the seasonal distribution of adenovirus with a lower proportion of positive cases in the Winter (Table 4.22).

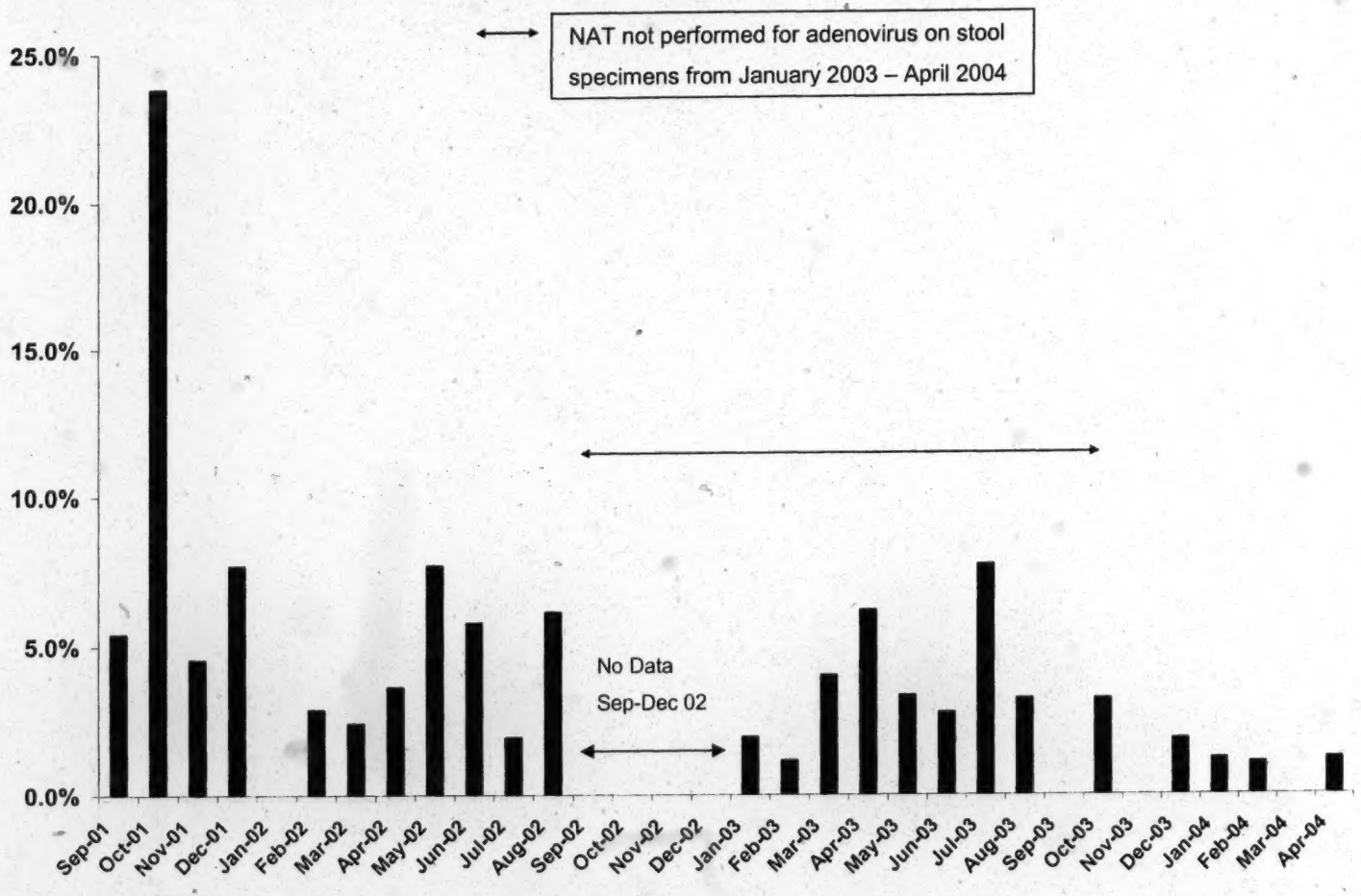
Table 4.22 Seasonal distribution of cases tested positive and negative for enteric adenovirus by nucleic acid amplification test (NAT) and/or electron microscopy (EM)*†

Season	No. of cases tested positive for adenovirus by NAT and/or EM (%)	No. of cases tested negative for adenovirus by NAT and/or EM (%)	Total
Autumn	8 (6.7%)	111 (93.3%)	119
Winter	17 (1.7%)	959 (98.3%)	976
Spring	14 (4.2%)	316 (95.8%)	330
Summer	14 (4.6%)	293 (95.4%)	307
Total	53 (3.1%)	1,679 (96.9%)	1,732

* Over the 28 month of the phase I and phase II of the study: Autumn encompassed a 4-month period, Winter a 13-month period, Spring a 5-month period and Summer a 6-month period

† χ^2=15.0, df=3, p<0.05 for the comparison of the distribution among the different seasons

Figure 4.7 Monthly distribution of the proportion of cases tested positive by nucleic acid amplification test and/or electron microscopy for enteric adenovirus (N=53)



4.4.3 Seasonality of astrovirus

There were a total of 61 cases of astrovirus and no astrovirus was identified from June to August in 2002 and 2003 as well as in March 2002, April 2003 and September 2003 (Appendix G). The monthly proportion of cases tested positive for astrovirus is shown in Figure 4.8. The two months with the highest positive rate of astrovirus were December 2001 and January 2004, at 15.4% and 11.0% respectively. There was significant difference in the seasonal distribution of astrovirus with no cases identified in the Summer (Table 4.23).

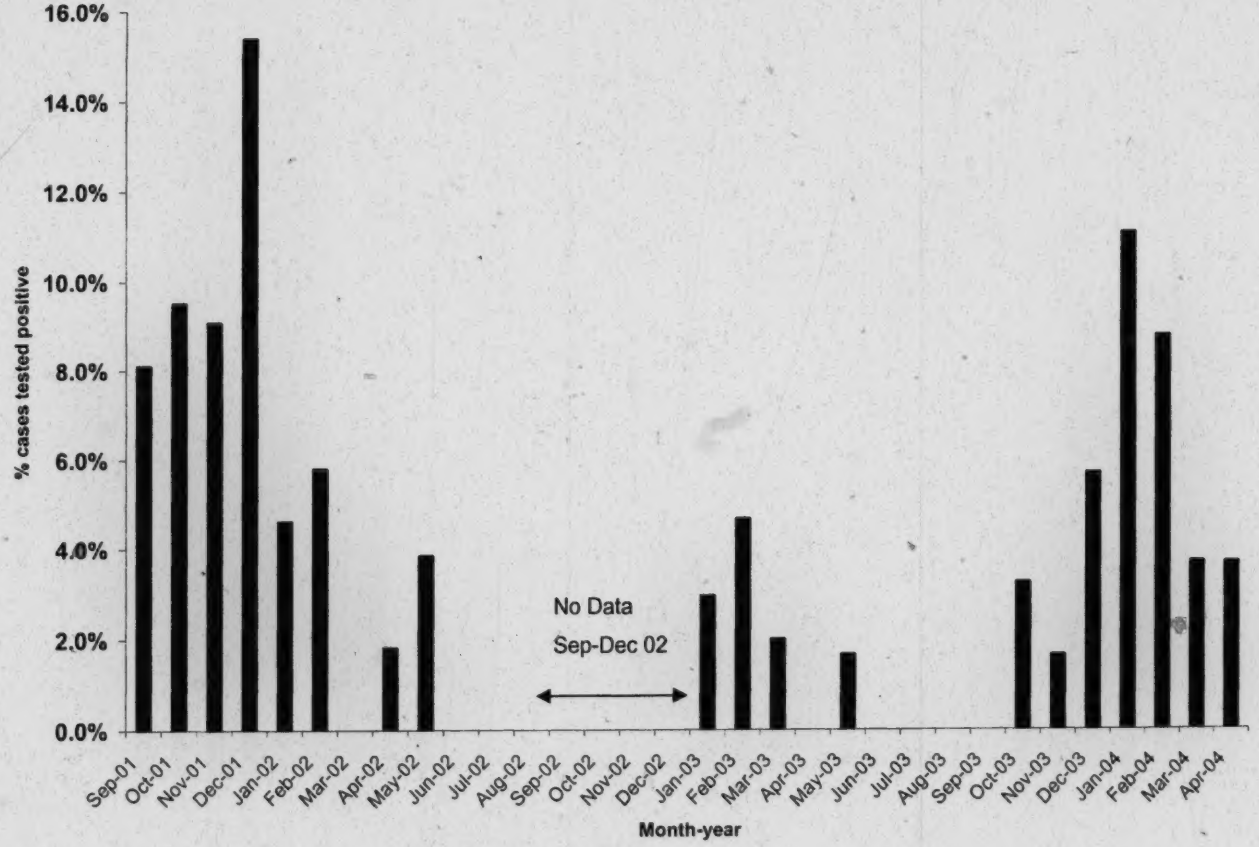
Table 4.23 Seasonal distribution of cases tested positive and negative for astrovirus by nucleic acid amplification test (NAT)*†

Season	No. of cases tested positive for astrovirus by NAT (%)	No. of cases tested negative for astrovirus by NAT (%)	Total
Autumn	6 (5.0%)	113 (95.0%)	119
Winter	48 (4.9%)	928 (95.1%)	976
Spring	7 (2.1%)	323 (97.9%)	330
Summer	0 (0.0%)	307 (100.0%)	307
Total	61 (3.5%)	1,671 (96.5%)	1,732

* Over the 28 month of the phase I and phase II of the study: Autumn encompassed a 4-month period, Winter, a 13-month period, Spring, a 5-month period and Summer, a 6-month period

† Fisher exact test, $p<0.001$ for the comparison of the distribution among the different seasons

Figure 4.8 Monthly distribution of the proportion of cases tested positive by nucleic acid amplification test for astrovirus (N=61)



4.4.4 Seasonality of norovirus

There were a total of 166 cases of norovirus detected and there were five months in the 28-month study period when there was no norovirus identified: September 2001, October 2001, June 2002, August 2002 and October 2003 (Appendix H). The monthly proportion of cases tested positive for norovirus is shown in Figure 4.9, and there was no obvious pattern of seasonal distribution for norovirus. However, with the χ^2 test, there was significant difference in the seasonal distribution of norovirus with a higher proportion of positive cases in Winter (Table 4.24).

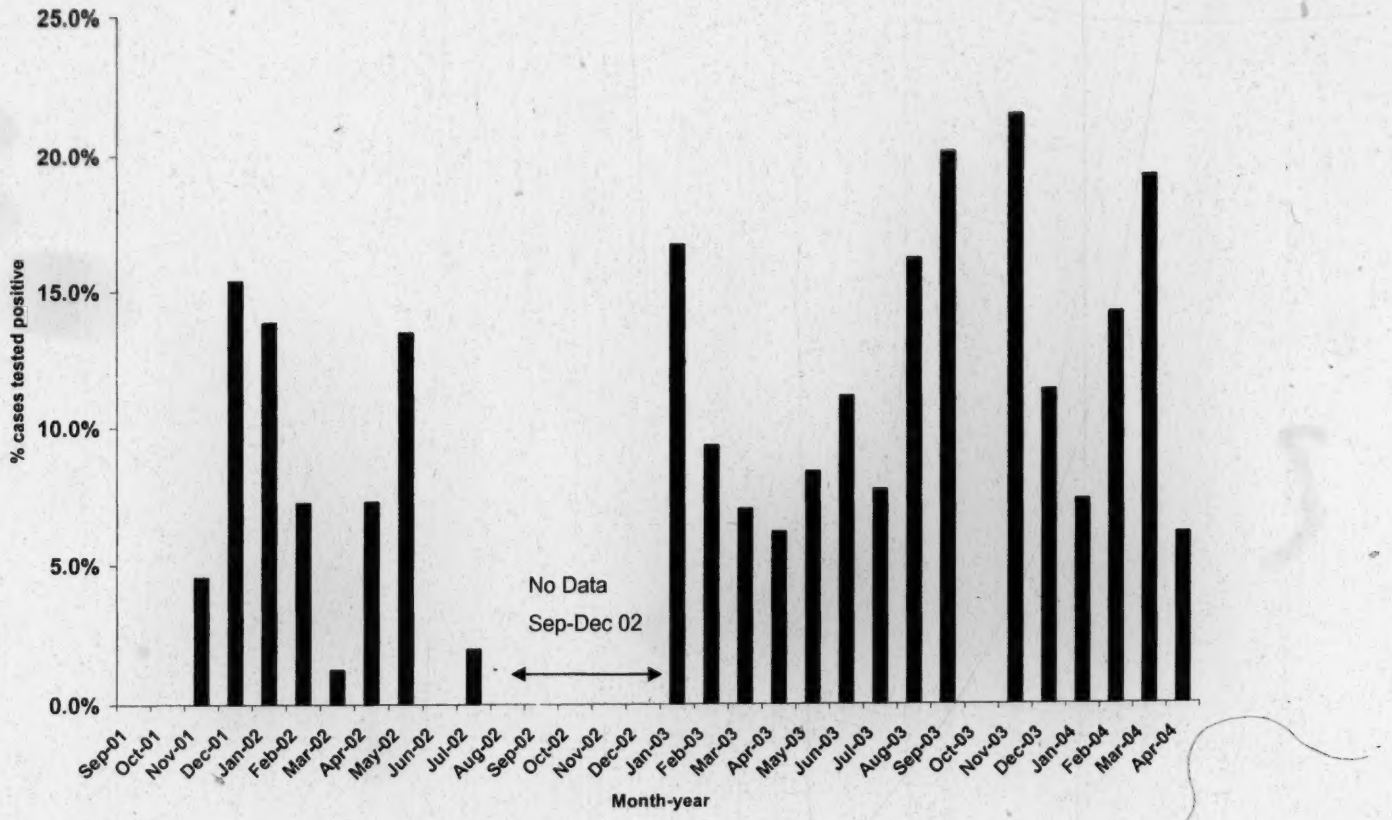
Table 4.24 Seasonal distribution of cases tested positive and negative for norovirus by nucleic acid amplification (NAT)*†

Season	No. of cases tested positive for norovirus by NAT (%)	No. of cases tested negative for norovirus by NAT (%)	Total
Autumn	6 (5.0%)	113 (95.0%)	119
Winter	116 (11.9%)	860 (88.1%)	976
Spring	26 (7.9%)	304 (92.1%)	330
Summer	18 (5.9%)	289 (94.1%)	307
Total	166 (9.6%)	1,566 (90.4%)	1,732

* Over the 28 month of the phase I and phase II of the study: Autumn encompassed a 4-month period, Winter, a 13-month period, Spring, a 5-month period and Summer, a 6-month period

† χ^2=15.0, df=3, $p<0.05$ for the comparison of the distribution among the different seasons

Figure 4.9 Monthly distribution of the proportion of cases tested positive by nucleic acid amplification test for norovirus (N=166)



CHAPTER 5 DISCUSSION AND CONCLUSION

Viral gastroenteritis is a major cause of morbidity and mortality in infants and children worldwide.[11,36,72,73,74,75] Traditional diagnostic methods using electron microscopy (EM) and viral culture to identify enteric virus have limited sensitivity and specificity. This is the first study in Northern Alberta that utilized both EM and nucleic acid amplification tests (NAT) to identify rotavirus, enteric adenovirus, astrovirus and norovirus in stool samples submitted for virological investigations in young children. While the hypothesis is that NAT would be more sensitive than EM for the detection of the enteric virus proved to be true for rotavirus, astrovirus and norovirus, it is interesting that NAT did not identify significantly more cases of adenovirus as compared to EM. After duplicate specimens were removed, rotavirus was the most commonly identified enteric virus, which is similar to previous findings in Finland.[21,55,56,57,58,59] With the use of NAT in this study, norovirus was identified for the first time as the second most common enteric virus in childhood gastroenteritis in Northern Alberta. The following discussions focus on 1) the analyses of the comparison between NAT and traditional assays, and 2) the potential bias and findings of the epidemiological data, 3) the seasonality of enteric viruses in the study.

5.1 Comparison of traditional viral investigations and nucleic acid amplification

Standard analysis of sensitivity, specificity or predictive values was not performed in this study because of the lack of a gold standard, as NAT and EM

have different strengths and requirements for detecting viruses, and a positive test by either assay is probably a true positive. While both NAT and EM are more likely to detect the virus in the early phase of gastroenteritis when viral excretion is high, the two methods have different requirements in terms of storage criteria and detection targets. Detection by EM is dependent on the preservation of viral morphology and the detection by NAT is based on the stability of nucleic acids in the samples and the absence of inhibitors.[76,77]

The NAT assays for each enteric virus used in this study have been validated according to the standards in published criteria and each test was performed with both positive and negative controls in the testing procedure.[78,79,63,80,69] The detection by EM was performed by diagnostic technologists who have been trained and have demonstrated competency in the area. The analysis in this study is based on the assumption that a positive result by either EM or NAT is a true positive and a specimen tested negative by both methods is a true negative.

An approach commonly used to compare diagnostic tests in the absence of a gold standard is discrepant analysis, which reclassifies cases based on concordant results of ancillary tests. This methodology has been criticized as biased and unscientific by various authors.[81,82] Alternative approaches such as latent class analysis with Bayesian modeling or composite reference design require knowledge and estimations of parameters beyond the scope of this study.[83,84,85,86] Another approach would be to assume samples tested positive by NAT or EM as true positives and estimate the sensitivity and specificity of each

assay based on the assumption. However, the estimated sensitivity and specificity will be biased especially for NAT because of the high correlation of the positive results with NAT.

The hypothesis of this study was that NAT would be more sensitive than traditional assays for the detection of four enteric viruses.[22,87,88] The McNemar analysis was used to identify if there were significantly more cases of specific enteric virus identified by NAT as compared to EM (and viral culture in the case of adenovirus). Kappa value provides an estimation of the agreement of two diagnostic assays for a specific enteric virus and can be defined as the "chance-corrected proportional agreement".[71] Since kappa value is dependent on both the performance characteristics of the diagnostic assays and the prevalence of the condition in question, only the strength of agreement between the two tests for specific virus can be discussed and the kappa value obtained for the different enteric viruses cannot be compared.

Among the enteric viruses, norovirus had the highest overall increase of detection by NAT (97.9%) and the kappa value comparing NAT for norovirus and EM detection as small round structured virus (SRSV) was very low. These findings are the results of both the high sensitivity of the NAT used in this study for norovirus and the poor sensitivity and specificity of EM for norovirus.[22] Astrovirus, similar to norovirus, is identified as SRSV by EM. While NAT detected a significantly higher number of astrovirus in phase II of the study, the kappa value between NAT and EM for astrovirus still indicated fair agreement between the two tests. The fact that the majority of SRSV identified by EM in

Northern Alberta were astrovirus is only recognized after the use of NAT. This finding confirms the low differentiability of EM as a diagnostic tool for SRSV as compared to NAT.

A higher proportion of samples tested positive by EM and negative by NAT for rotavirus in phase II as compared to phase I. The most obvious explanation for the loss of sensitivity of NAT would be the change in study design and storage procedure for the two phases of the study with loss of specimen integrity. On the other hand, this would not explain why a similar trend was not observed for norovirus and astrovirus. Emergence of new rotavirus genotype has been reported in several countries[89,90,91,92,93] and the sensitivity of NAT is very dependent on the choice of target and primer sequence. Thus, another possible explanation for the increase in the positive EM and negative NAT specimens in phase II is a shift of the circulating strains of rotavirus in Northern Alberta with variation in genetic sequence that has affected primer-binding and amplification. To test the hypothesis, a research project has been initiated at the Provincial Laboratory for Public Health (ProvLab) to subtype the strains of rotavirus identified in this study.

The primer sequence for adenovirus in this study was chosen to be specific for serotypes 40 and 41 to avoid detection of non-enteric adenovirus that sometimes have prolonged excretion in stool samples.[18] The fact that no EM positive specimens tested negative by NAT suggesting that adenovirus serotypes 40 and 41 were the predominant circulating enteric adenovirus in Northern Alberta as in other studies.[94,95] On the other hand, this highly specific NAT had

little increase in detection of adenovirus as compared to EM. Thus NAT was not performed for adenovirus during phase II.

In terms of the two traditional viral studies for adenovirus, a low concordance rate between EM and viral culture for adenovirus in this study was expected because of the poor growth of adenovirus serotypes 40 and 41 in culture as previously described.[18] However, it has been shown that some of these enteric adenoviruses can be propagated by culture at a higher rate than originally believed.[96] In this study, 46% of the EM positive samples that were also positive by viral culture were confirmed to be adenovirus serotypes 40 by NAT supporting the observation that some enteric adenovirus can be propagated in culture. Most of the adenovirus isolated by culture in this study tested negative by NAT (which is of comparable sensitivity with EM) and were presumably non-enteric adenovirus. Since this study confirmed that addition of viral culture to EM or NAT had a low yield for enteric adenovirus and often yielded 'false-positive' results, the diagnostic algorithm at ProvLab was changed in July 2003 to stop routine culture for stool.

An additional advantage of NAT as compared to the traditional methods in the current study was the identification of mixed viral infections. Theoretically, mixed infection can be recognized by identification of multiple viral morphologies by EM, but in reality, this rarely happens because only a very small amount of stool sample was being examined at one time. No mixed infections were identified by using only EM in the current study. The 6.5% rate of mixed infections in the current study falls within the range of 0.04% to 18% (median 8%)

identified in previous studies that used NAT.[57,97,98,99,100,101,102,103,101,104,105,106] The variations in the rate of mixed infection in different studies was likely related to the population being studied and the different combinations of detection methods including EM, EIA and NAT. Only previous studies from Finland used NAT to detect all four enteric viruses[57,63,69,80], but the current study used real-time NAT which is even more sensitive than the conventional NAT used in the Finnish study. Most previous studies identified rotavirus as the most frequently found virus in mixed infection. The equal proportion of rotavirus and norovirus in mixed infection in the current study was likely reflective of the fact that they were the two most commonly identified viruses in the study.

In summary, the difference in the proportional increase of detection by NAT for various enteric viruses illustrated the importance of the sensitivity of EM for each respective virus and the sensitivity of NAT for each virus based on primers selection, amplification conditions, and the type of platforms selected for NAT.

5.2 Epidemiology of childhood gastroenteritis in Northern Alberta

To get a better estimation of the disease burden of enteric virus in Northern Alberta as identified by EM and NAT, duplicate specimens <15 days apart were removed from the case-based analysis. Other studies have used a duration of seven to 14 days to separate different episodes of gastroenteritis.[107,108] A duration of 15 days was chosen for this study because of the presumed high sensitivity of NAT for detection of asymptomatic shedding.

The fact that it could not be confirmed that all stool samples came from patient with clinical gastroenteritis in the current study might explain the lower overall detection rate of enteric virus (36.2%) as compared to the ~60% detection in the Finnish and Beijing studies where NAT was also used for detection.[57,106]

Rotavirus was the most common enteric virus in childhood gastroenteritis in Northern Alberta. Using the average annual number of children less than seven years old in Northern Alberta from 2001-2004 (courtesy of Alberta Health and Wellness Surveillance Branch), and the average annual number of cases for each enteric virus detected in the 28-month study, the number of cases per 10,000 per year for rotavirus was 9.0 per 10,000 children per year, followed by 3.8 per 10,000 children per year for norovirus, 1.6 per 10,000 children per year for enteric adenovirus, and 1.4 per 10,000 children per year for astrovirus (Table 5.1), It is important to have accurate estimation of the disease burden for rotavirus since clinical trials are underway for a new rotavirus vaccine.[109,110] Initial data from EM suggested a significant increase in the proportion of cases tested positive for rotavirus in phase II of the study, an observation that was not supported by the combined NAT and EM data. With the higher number of specimens tested positive by EM and negative by NAT in phase II, it is possible that the rotavirus disease burden was underestimated in phase II due to new variants of rotavirus and decreased detection by NAT.

Table 5.1 Annual rate of the enteric viruses in children less than seven years old in Northern Alberta

	2001	2002	2003	2004
No. children less than seven years old per annum in Northern Alberta*	179,331	178,810	178,149	177,338
Average annual number of children less than seven years old in Northern Alberta	178,407			
	Rotavirus	**Enteric adenovirus**	**Astrovirus**	**Norovirus**
No. of cases of enteric virus identified in the 28-month study	374	28†	58	158
Average no. of cases of enteric virus in a 12-month period	160.3	28.0†	24.9	67.7
Annual rate of enteric virus per 10,000 children less than seven years ole	9.0	1.6	1.4	3.8

* Courtesy of Alberta Health and Wellness Surveillance Branch

† Only 12 month of data included as NAT was not performed in phase II for adenovirus.

Norovirus was only recognized as the second most common pathogen in sporadic childhood gastroenteritis by the use of NAT. The proportion of positive norovirus cases in phase II was significantly higher than that in phase I. This increase might be due to a higher sensitivity of the TaqMan assay as compared to the LightCycler assay, or changes in circulating norovirus strains and host susceptibility.[69,80,111,112,113] A similar increase in annual disease burden has been reported in norovirus outbreaks in Northern Alberta and other countries for the same time period (Appendix J).[114,115,116] Delineating the genetic relationships

between norovirus in childhood gastroenteritis and in outbreaks might further the understanding of the circulatory pattern of this important pathogen. There is a project in progress at ProvLab to examine the molecular epidemiology of norovirus strains in these two settings.[117]

Young age was associated with the detection of enteric virus in this study as previously described.[27,107,99,100,118,119,120] Infection by rotavirus produces protective immunity early in life, but it might take several infections before the development of consistent immune response.[16,121] The immune response to norovirus is still under study and is hindered by the absence of a viral culture system.[122,123,124] The finer categorization of age group used in this study allowed the identification of a low risk period in neonates less than three months of age for viral gastroenteritis, which is likely due to the protective effects of transplacental antibodies and breast feeding.[1,125] The odds ratios of identifying enteric virus, in particular rotavirus, in stool samples were highest from six to 18 months of age, a susceptible period before the acquisition of natural immunity to the various enteric viruses. On the other hand, the infection of norovirus appears to be more evenly distributed from three to 24 months of age. There was no obvious association of adenovirus and astrovirus with age which might be due to the small number of cases. Gender as a significant associated factor was only identified with norovirus with cases being predominant in females, but the lower limit of the confidence interval for the odds ratio of 1.4 was only 1.02, suggesting further studies are needed. It is possible that there is gender difference in blood group antigens or other cell receptors that are important for norovirus infection.

The association between the virologic tests being requested by family physicians/general practitioners and an overall higher rate of enteric virus is likely confounded by the high rate of rotavirus cases in Mistahia and a high proportion of submitters from family/general practice compared to pediatricians in Mistahia. This finding was confirmed by the fact that only young age and Mistahia were identified as significant factors associated with rotavirus infection in the multivariate analysis for rotavirus.

Certain geographic regions in Northern Alberta were associated with a higher rate of specific virus such as Mistahia with rotavirus and Northern Lights Region with enteric adenovirus. The median age of the cases from Mistahia (1.35 year) is significantly higher than the other regions (0.85 in Capital Health, 1.0 in Northern Lights). A possible explanation of the higher proportion of rotavirus case in Mistahia is that Capital Health and Northern Lights had more stool samples submitted from neonates less than three months of age who are at a lower risk for rotavirus infection. On the other hand, even though norovirus infections are also associated with the age group of three months to less than 48 months old, cases of norovirus were evenly distributed in Northern Alberta. An alternate explanation for the association of rotavirus and adenovirus with specific geographic regions is the presence of undetected outbreak activity in the regions. An automated, laboratory-based system to detect outbreaks has been in use in the Netherlands.[126] It would be ideal to set up a similar alert system in Northern Alberta using results generated by the sensitive NAT.

5.3 Biases in the epidemiological analysis

This study was based on NAT and EM of stool specimens submitted for virological investigations at ProvLab, which is the diagnostic laboratory that performed all the virological testing for stool specimens from Northern Alberta. Based on the assumption that the submitting physicians had requested testing for virus in the stool samples because of their clinical suspicions for viral gastroenteritis, the specimens would be representative of sampling from children with symptomatic illness. However, laboratory-based surveillance represents a very small portion of clinical gastroenteritis cases, as not all symptomatic patients seek health care and not all physicians submit specimen for virological studies. The inherent bias is likely towards stool samples being submitted in more severe cases. Using a community-based surveillance to capture all gastroenteritis cases will provide a more complete estimation of disease burden, but considerable resource is needed to establish a sentinel surveillance system with families and clinics. Despite this limitation of laboratory-based surveillance, the importance and utility of laboratory-based data for gastroenteritis illness has been discussed because of the underreporting of clinical-based surveillance.[127,128]

Phase I and phase II of the study used different approaches to accessing and storing the stool specimens for NAT. The prospective selection of specimens from the appropriate age groups in phase I was less efficient than the retrospective approach of storing all specimens with later selection of specimens from the appropriate age groups in phase II as virology staff did not always remember to select and save specimens from appropriate age groups for the

study. The lower inclusion rate of stool samples in phase I of the study might be a confounding factor for the significant difference observed for the proportion of included cases among the different regions and among the various types of clinical practice in phase I. On the other hand, there should be no bias in terms of the geographic distribution or the type of clinical practice on the overall estimation of disease burden in the cases included in the final analysis because the only significant difference between included and excluded cases was the higher rate of inclusion in phase II of the study by multivariate binary logistic regression.

Only a small percentage of cases had unknown gender (1.3%) and were submitted by physicians of unclassified type of practice (5.5%). Geographic regions of the majority of cases were assigned by the health region of the physicians because the patients' residence was not available. An assumption was made that patients seek care in proximity of their residence and that the data would still be representative of where the enteric virus was circulating.

5.4 Seasonality of enteric viruses

In terms of seasonality analysis, this study is limited by a loss of continuity with the four-month gap between phase I and phase II of the study. On the other hand, the Winter predominance of rotavirus and norovirus was still impressive. A similar type of seasonal pattern has been found in outbreak settings for norovirus and in other epidemiological studies in the United States, Finland, England, Argentina and most recently in China and Korea.[57,98,102,129,130,131,132] Only a few studies from Spain, Japan, England and Wales have shown unusual patterns for

rotavirus and norovirus with the absence of a Winter peak.[133,134,135] Even though the total number of cases of astrovirus and enteric adenovirus was comparatively small in this study, astrovirus showed a distinct seasonal pattern with no case in the Summer and enteric adenovirus showed a reverse pattern with a lower rate in the Winter. Clear seasonality has also been observed for other viruses such as influenza and other respiratory viruses, but the mechanism remains unknown.[136,137,138,139] Using genetic sequencing and molecular epidemiology to study the enteric viruses will be the important next step to further our knowledge in the circulatory patterns of enteric viruses.

5.5 Conclusion

This is the first study to review the disease burden of different enteric viruses in young children in Northern Alberta. While the study is limited by the lack of case ascertainment with clinical data, the use of NAT provides enhanced surveillance with a significantly higher rate of detection as compared to traditional EM for three of the four common enteric viruses in childhood gastroenteritis. The disease burden for all enteric viruses is likely underestimated in this laboratory-based surveillance study and might be biased towards more enteric viruses that cause more severe illness. Rotavirus and norovirus were found to be the predominant enteric viruses and the importance of norovirus in childhood gastroenteritis is only recognized with the use of NAT. Accurate estimation of disease burden is critical for surveillance and disease prevention planning, such as the introduction of rotavirus vaccine.[140] Improving the diagnostic tools will provide better surveillance data to understand the circulation of enteric virus. An

important application will be to identify a link between the norovirus strains in sporadic gastroenteritis and institutional outbreaks and find potential prevention strategies.

Understanding the seasonality of enteric virus is also important for healthcare resource planning. The cold weather predominance of rotavirus, norovirus and astrovirus infection coincides with respiratory virus season which also targets young infants and has major impacts on healthcare services. There is annual variation of disease rate and further information will help to predict patterns. Anticipatory programs such as education for home-based oral dehydration before Winter might reduce unnecessary emergency and physician visits and decrease the winter pressure for the hospitals.

Public health surveillance is the ongoing, systematic collection, analysis, interpretation, and dissemination of data regarding a health-related event for use in public health action to reduce morbidity and mortality and to improve health.[140] Enteric viruses are important cause of gastroenteritis in young children and advanced molecular diagnostic assays that improve the identification of enteric viruses is the first step towards better understanding of this important disease.

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Appendix A Surveillance study identifying etiologies of gastroenteritis

Author	Year	Journal	Study design and patient population	Definition of gastroenteritis (if available)	Diagnostic methods	Findings
Huilan S[7]	1991	Bull WHO	Case-control 0-35 months children from China, India, Mexico, Myanmar, Pakistan. Case control (2 year) (N=3,640 cases, N=3,279 controls)	History of diarrhea for 72 hours or less	Routine bacteriology culture and EM	68% had pathogen identified: 48% bacterial, 23% viral, 3% *G. lamblia*. Overall most common pathogens associated with disease were rotavirus, *Sheigilla* and ETEC
Caprioli A[141]	1996	Pediatr Infect Dis J	<10 years admitted or outpatients from 6 hospitals in Italy (1 year)	≥3 loose stool per day	Routine bacteriology culture and rotavirus and adenovirus by	Significant etiologies identified in 59% of cases as compared to controls: rotavirus (23.6%), Salmonella (19.2%) and

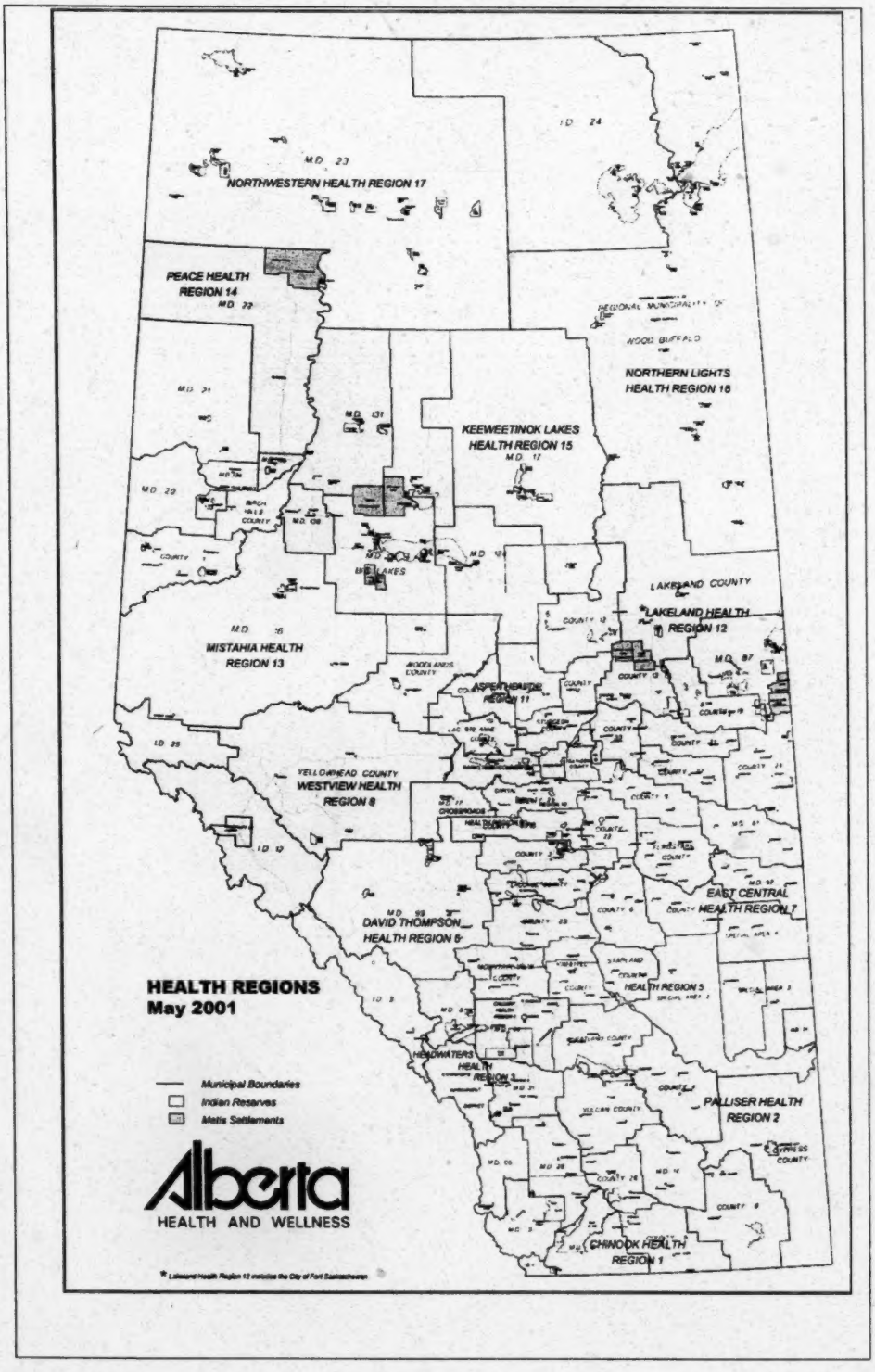
			(N=618 cases, N=135 controls)		EIA	Campylobacter (7.9%)
Rohner P[142]	1997	J Clin Micro	Lab-based surveillance Geneva (4 year) (913,965 specimens from 7,124 patients)	Not specified	Specimens that were positive for bacterial and positive by EIA for rotavirus	For patients ≤6 years hospitalized ≤3 days: rotavirus (11.9%), bacteria (8.8%)
McIver CJ HC[143]	2001	Pathology	<6 years admitted or outpatients in Sydney (1 year) (N=412)	≥3 loose stool per day for less than 15 days with no other illness	Routine bacteriology culture, EM and rotavirus, adenovirus, astrovirus by EIA, norovirus by RT-PCR	Etiologies identified in 33% of cases: rotavirus (40%), adenovirus (26%), astrovirus (12%) and *Campylobacter* (12%), *Salmonella* (10%), Giardia lamblia (<1%)
Maltezou HC[144]	2001	J Infect	Median age: 2 years (range: 1 month – 12.5 years) presented to emergency with	≥3 loose stool per day for less than 15 days with no other illness	Routine bacteriology culture and rotavirus and adenovirus by EIA	Rotavirus (14%), *Salmonella* (9%), *Campylobacter* (7.5%), (adenovirus 4.5%). Two peaks of rotavirus (Feb & Aug)

			acute diarrhea in Greece. (1 year) (N=132)			
Youssef M[145]	2000	FEMS Immuno Med Micro	0-5 years hospitalized with acute diarrhea in Jordan (two 4-month period May-Aug) (N=265)	3 or more loose stool in preceding 24 hours	Routine bacteriology culture, PCR for various *Escherichia coli* as confirmation, examination for parasites, EIA for rotavirus and EM	Rotavirus (32.5%), Enteropathogenic coli (12.8%), enteroaggregative coli (10.2%). No adenovirus, no small round structured virus by EM
De Wit MAS[107]	2001	Clin Infect Dis	Case-control children and adults presented to General practice with diarrhea in Netherlands (3	Diarrhea with ≥2 additional symptoms, vomiting with ≥2 additional symptoms, ≥3 loose stool in	Routine bacteriology culture, examination for parasites, EM for virus, EIA for	In terms of significant pathogens identified as comparing case to controls: Cases <1 year: almost all pathogens were virus (rotavirus and norovirus most common); case 1-4 years:

			years) (N=574 cases, N=857 controls)	preceding 24 hours with an episode of gastroenteritis to be preceded by 2-week symptom free period	rotavirus, astrovirus and adenovirus. RT-PCR for norovirus and sapovirus	majority was virus (rotavirus most common); Cases 5-14 years: *Campylobacter* most common followed by *G. lamblia*. Overall viruses accounted for 30-50% of cases for children <5 years
Marie-Cardine A[97]	2002	Clin Infect Dis	1-35 months hospitalized with acute diarrhea in France (two 3-month period Dec-Feb) (N=438)	Increase of loose stool to more than normal for a period of 15 days	Routine bacteriology culture, EIA for rotavirus, astrovirus and adenovirus. RT-PCR for astrovirus, norovirus and sapovirus	Virus identified in (37%): rotavirus (17.3%), norovirus and sapovirus (7.3%), astrovirus (6.8%), adenovirus (0.7%), *Salmonella* (0.007%). 5% had dual infection: rotavirus, most common, followed by astrovirus and norovirus/sapovirus
Denno DM[146]	2005	Pediatr Infect Dis J	<21 years presented to ambulatory pediatric clinics	Not specified	All were tested by routine bacteriology culture, 56%	Children <2 years: 31.8% virus, 2.5% bacterial, Children: 2-21 years 6.5% virus 9.2% bacterial

			in Seattle with mean age of 18.7 months (12 months at one site and 22 months at another site) (N=226)		for examination for parasites, only 33.2% tested by EIA for rotavirus, astrovirus and adenovirus.	

Appendix B Regional designation for Alberta May 2001 to April 2003



Appendix C Primers and probes for the nucleic acid amplification tests for the four enteric viruses: rotavirus, adenovirus, astroviurs and norovirus

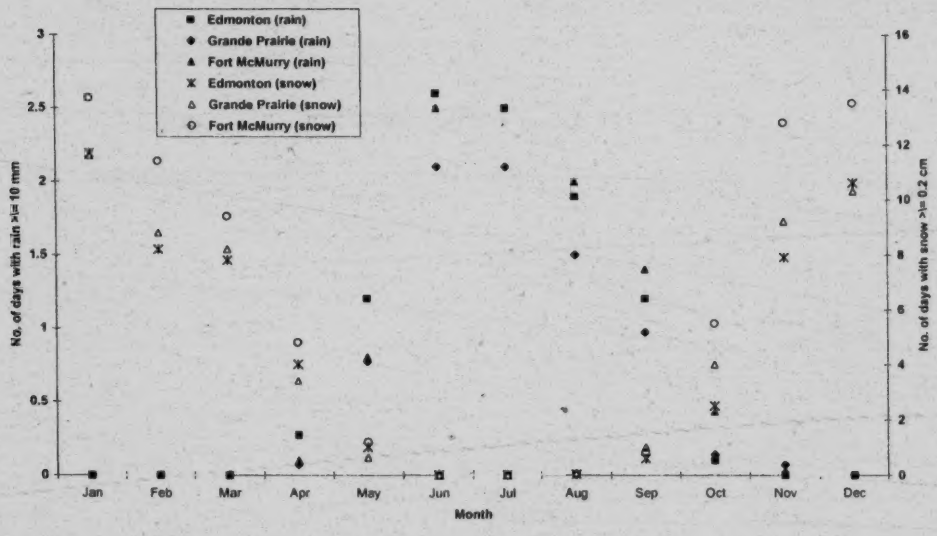
Virus (Genbank accession #)	Name of primer	Sequence	Length (base pair)	Position	Amplicon size (base pair)	Detect capacity	Reference
Rotavirus (X81436)	NSP3-F	accatctacacatgaccctc	20 (+)	963 - 982		Detect all group A rotaviruses	Pang et al. 2004[63]
	NSP3-R	ggtcacataacgcccc	16 (-)	1034 - 1049	87		
	NSP3p	atgagcacaatagttaaaag ctaacactgtcaa	33 (+)	984 - 1016			
Enteric adenovirus (D13781)	Ead-F	ccctacttcacctactct	18 (+)	2225 - 2242		Detect serotype 40 and 41	Pang, unpublished data
	Ead-R	cattgagccacgttgt	16 (-)	2397 - 2412	187		
	Ead-probe-FL	ggtgtccatcatgtttgactcct	23 (+)	2296 - 2318			
	Ead-prob-LC	gtgagttggcctggcaat	18 (+)	2321 - 2338			
Astrovirus (Z25771)	Ast-beg	accgtgtaaccctcctctc	19 (+)	6495 - 6513		Detect all serotype except type 4	Saito et al. 1995[65]
	Ast-end	tcctactcggcgtggccgc	19 (-)	6717 - 6735	241		

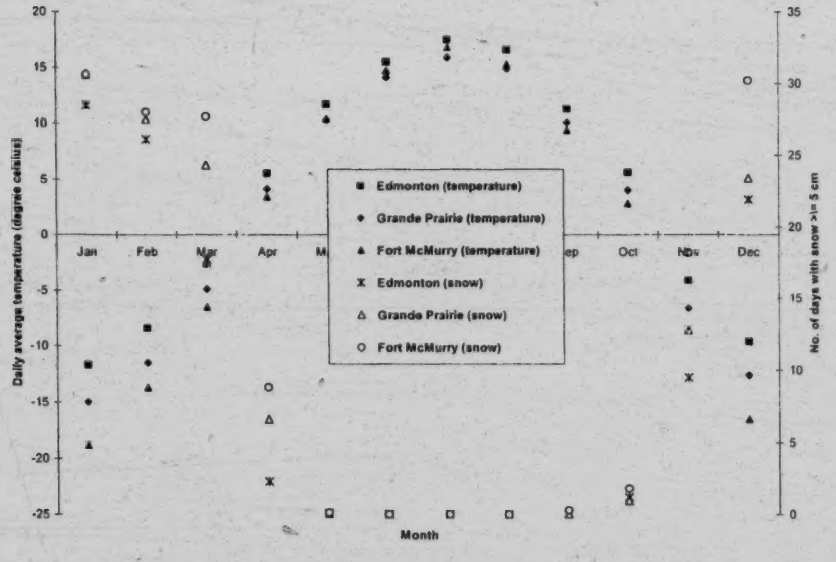
Norovirus GI (M87661)	G1-F	cgytggatgcgnttycatga	20 (+)	5291 - 5310		Mix could detect all norovirus GI&GII	Pang et al. 2005[69]
	G1-R	cttagacgccatcatcattyac	22 (-)	5354 - 5375	85		
	G1a-Probe	agatygcgatcycctgtcca	20 (-)	5321 - 5340			
	G1b-Probe	agatcgcggtctcctgtcca	20 (-)	5321 - 5340			
Norovirus GII (AF145896)	G2-F	cargarbcnatgttyagrtggatgag	26 (+)	5003 - 5029			
	G2-R	tcgacgccatcttcattcaca	21 (-)	5080 - 5100	98		
	G2-Probe	tgggagggcgatcgcaatct	20 (+)	5048 - 5067			

Appendix D Canadian climate averages (1971-2000) for Edmonton, Grande Prairie and Fort McMurray with data extracted from Environment Canada

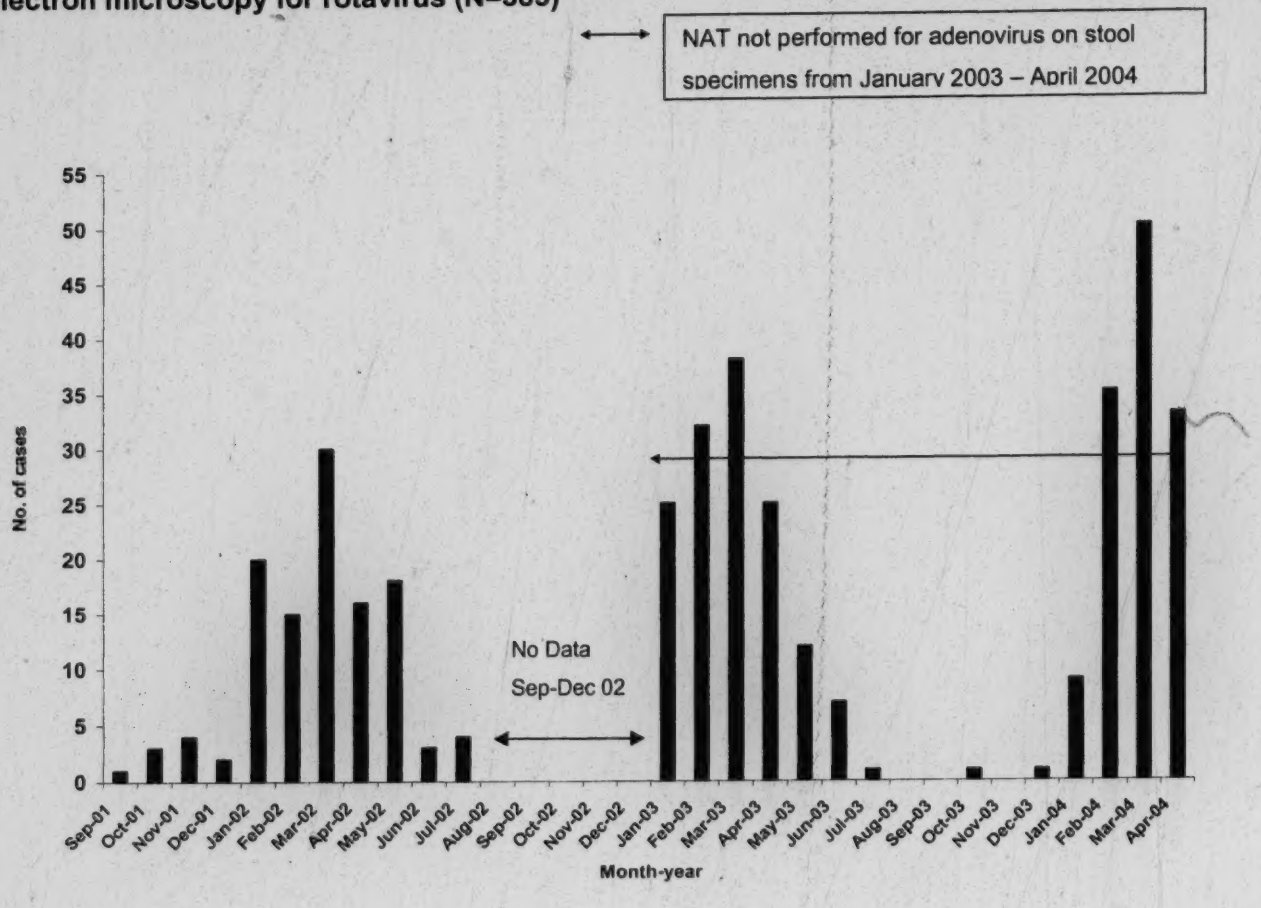
Taken from

http://www.climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html

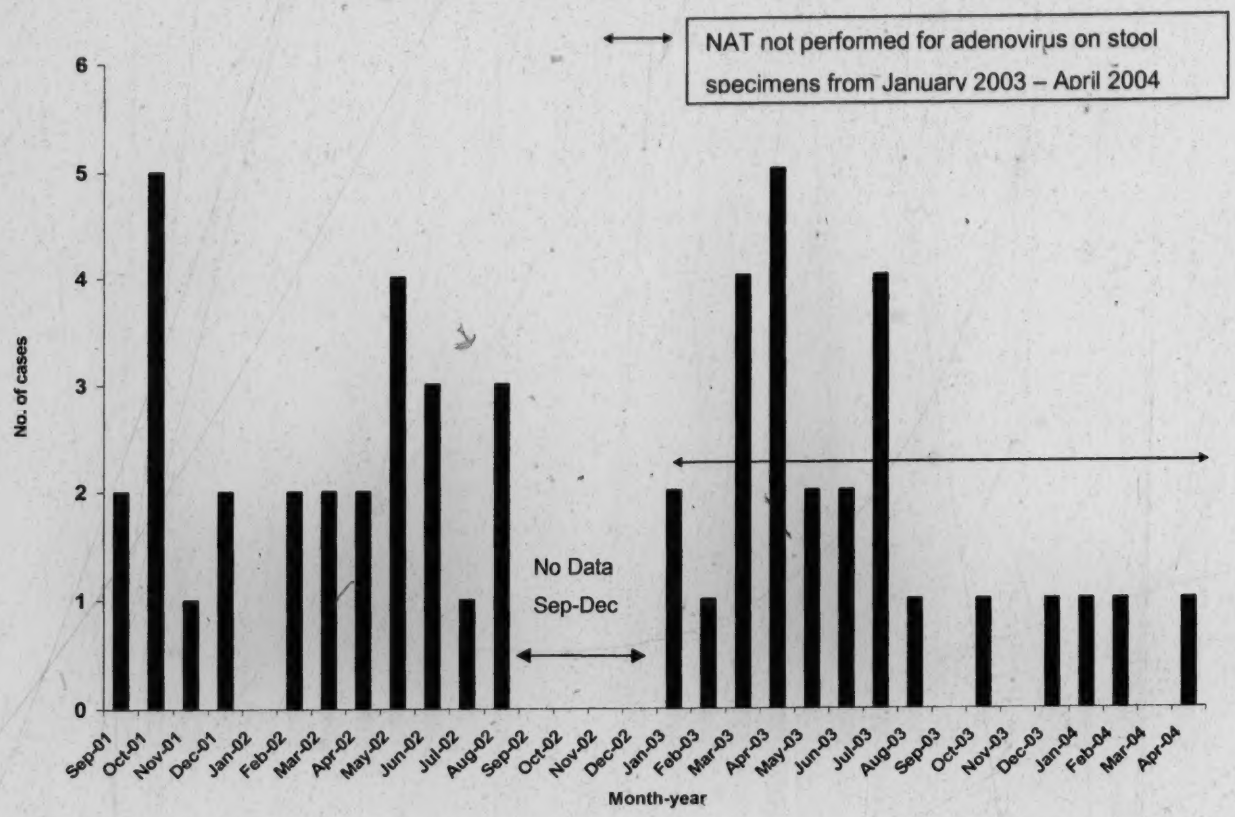




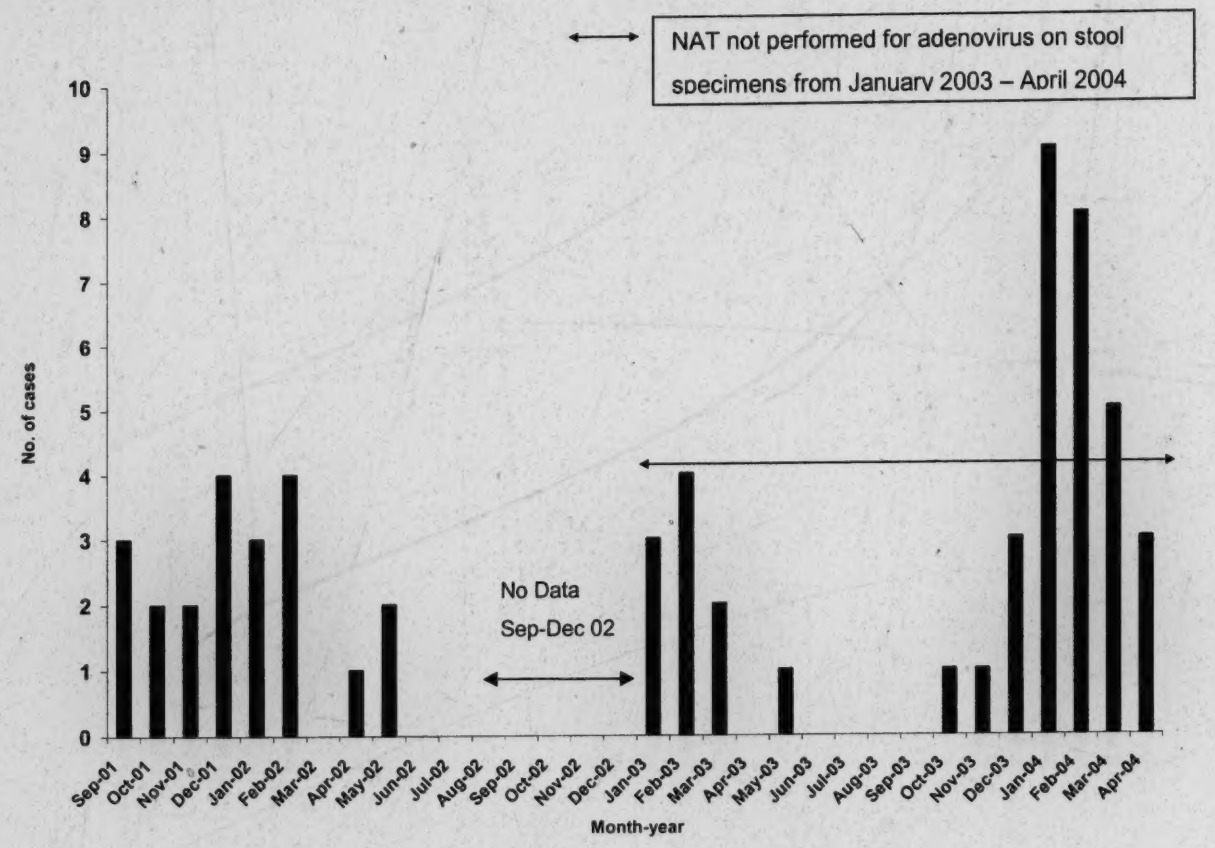
Appendix E Monthly distribution of the number of cases tested positive by nucleic acid amplification test and/or electron microscopy for rotavirus (N=385)



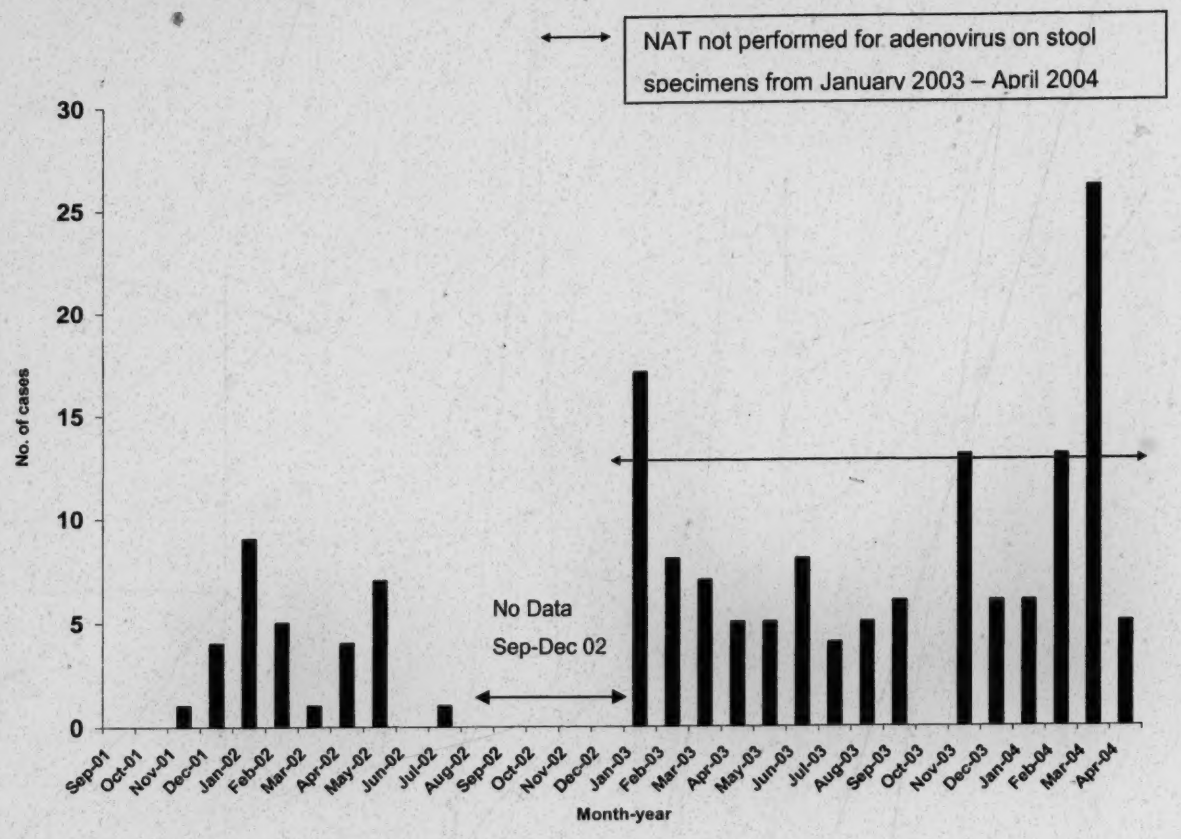
Appendix F Monthly distribution of the number of cases tested positive by nucleic acid amplification test and/or electron microscopy for enteric adenovirus (N=53)



Appendix G Monthly distribution of the number of cases tested positive by nucleic acid amplification test for astrovirus (N=61)



Appendix H Monthly distribution of the number of cases tested positive by nucleic acid amplification test for norovirus (N=166)



Appendix I Epidemiological studies on enteric viruses in children using nucleic acid amplification tests

Author	Year	Journal	Study design and patient population	Definition of gastroenteritis (if available)	Diagnostic methods	Findings with focus on enteric virus
Bon F[99]	1999	J Clin Micro	Case-control: 0-13 years children seen at clinic or Centre Hospitalier Universitaire for gastroenteritis, Dijon, France (27 months Dec 95 to Feb 98) (N=414 cases, N=50 controls)	Not specified	EIA for rotavirus adenovirus and astrovirus, RT-PCR for calicivirus	Virus identified in 72.7% of cases, 10% of controls and dual infection in 16.7% of cases. Of all the cases: rotavirus 60.8%, calicivirus 14%, astrovirus 6.3%, adenovirus 6.3%. Of the controls: astrovirus 2% (1 sample) and rotavirus 10% (5 samples). Of the mixed infections, rotavirus was most common followed by caliciviruses. Median age for rotavirus, adenovirus,

						astrovirus and calicivirus: 11, 15, 34 and 14.8 months respectively (p>0.05)
Mitchell DK[108]	1999	J Infect Dis	Young infants attending 8 child care centres in Norfolk, Virginia were monitored for gastroenteritis (12 months) (N=179 children, 928 samples)	Passage of unformed stool with at least twice the usual daily frequency ≥2 days and separated from previous diarrhea by ≥7 days	RT-PCR for astrovirus	In all the samples, 5.8% positive for astrovirus
Pang XL[57]	2000	J infect Dis	Randomized blinded placebo trial of rotavirus vaccine: 2,398 recruited children (<2 years) in	≥3 loose stool in 24 hours	EIA and RT-PCR for rotavirus, RT-PCR for astrovirus, norovirus,	Virus identified in 61% and mixed infections in 10% of cases. Of the cases tested: Rotavirus 28.9% (241 of 832), norovirus 20.2% (158 of 783), astrovirus 8.8% (71

			Tampere, Finland with prospective follow-up of gastroenteritis (12 months) (N=832 cases of gastroenteritis, not all cases tested for all viruses)		sapovirus, DNA PCR for adenovirus	of 811), adenovirus 6.3% (41 of 811), sapovirus 9.3% (72 of 776). Rotavirus, astrovirus and sapovirus peaked between March and May, norovirus peaked around December, adenovirus throughout the year.
O'Ryan ML[147]	2000		Case control: ≤5 year-old patient with gastroenteritis seen at health care facilities and clinics in Santiago, Chile (32 months Feb	Not specified	Norovirus testing only by RT-PCR	In total 8% positive for norovirus in cases and 0.8% in control. Decrease from 16% in 1997 to 2% in 1999

			97 to Sep 99) (N=1,787, 248 samples tested in study and 162 control)			
Farkas T[53]	2000		Birth cohort study: 0-24 months monitored for gastroenteritis in Mexico City (24 months) (N=115 cases, N=66 controls)	Not specified	Norovirus testing only by RT-PCR	In total 13% positive for norovirus in cases and 7% in control. All cases <12 months old with 87% between 6-12 months of age
Mustafa H[54]	2000	J Clin Micro	Specimens from <5 years-old patients admitted at Royal Children's	≥3 loose stool per day for less than 15 days with no other illness	Routine diagnostic tests for bacteria, rotavirus and	Etiologies (bacterial and viral) identified in 77% of cases. Of all cases: rotavirus (65.2%), astrovirus (3.0%),

			Hospital in Melbourne, Australia (3 years) (N=1,327)		adenovirus. Astrovirus tested by RT-PCR if sample tested negative for rotavirus	adenovirus (4.1%) and bacterial (6.3%). Majority of astrovirus occur in May to August (late Autumn and Winter) but seasonal peak not observed for all years. Majority of infected children <2 years
McIver CJ HC[143]	2001	Pathology	<6 years admitted or outpatients in Sydney, Australia (1 year) (N=412)	≥3 loose stool per day for less than 15 days with no other illness	Routine bacteriology culture, EM and rotavirus, adenovirus, astrovirus by EIA, norovirus by RT-PCR	Etiologies (bacterial and viral) identified in 33% of cases. Of all cases: rotavirus (13.5%), adenovirus (8.7%), astrovirus (4.1%) and *Campylobacter* (3.9%), *Salmonella* (3.3%), Giardia lamblia (<1%)
De Wit MAS[107]	2001	Clin Infect Dis	Case-control children and	Diarrhea with ≥2 additional	Routine bacteriology	Overall rotavirus 8% and norovirus 7.5% positive

			adults presented to General practice with gastroenteritis in Netherlands (3 year) (N=574 cases, N=857 controls)	symptoms, vomiting with ≥2 additional symptoms, ≥3 loose stool in preceding 24 hours with an episode of gastroenteritis to be preceded by 2-week symptom free period	culture, examination for parasites, EM for virus, EIA for rotavirus, astrovirus and adenovirus. RT-PCR for norovirus and sapovirus	rate. Significant pathogens identified after comparing case to control were: Cases <1 year: almost all pathogens were virus (rotavirus and norovirus most common); case 1-4 years: majority was virus (rotavirus most common); Cases 5-14 years: *Campylobacter* most common followed by *G. lamblia*. Overall viruses accounted for 30-50% of cases for children <5 years
Buesa J[26]	2002	J Clin Micro	Samples submitted from children <5 years to laboratory Valencia, Spain	Not specified	EIA for rotavirus, adenovirus and astrovirus.	Of all cases: rotavirus (25.3%), norovirus (14.2%), astrovirus (3.2%), adenovirus (3.2%).

			(24 months) (N=310)		RT-PCR for norovirus	
Martinez N[148]	2002	J Med Virol	Samples submitted from children <2 years to Children Hospital of Mendoza, Argentina (42 months: Jul 1995 to Dec 1998) (N=941)	Not specified	EIA and RT-PCR for norovirus	Of all cases: norovirus (2%). Zero specimen tested by EIA as positive.
Marie-Cardine A[97]	2002	Clin Infect Dis	1-35 months hospitalized with acute diarrhea in France (two 3-month period Dec-Feb) (N=438)	Increase of loose stool to more than normal for a period of 15 days	Routine bacteriology culture, EIA for rotavirus, astrovirus and adenovirus.	Virus identified in 37% and dual infections in 5.4% of cases. Of all cases: rotavirus (21.5%), astrovirus (10.2%), norovirus and sapovirus (9.8%), adenovirus

					RT-PCR for astrovirus, norovirus and sapovirus	(~1.0%), *Salmonella* (<1.0%). For dual infection: rotavirus, most common, followed by astrovirus and norovirus/sapovirus
Bereciartu A[98]	2002	J Clin Virol	<3 year outpatient with gastroenteritis seen at Ricardo Butierrez Children's Hospital of Buenos Aires, Argentina (12 months) (N=66 randomly selected cases from 659 children)	≥3 loose stool in <5 days	Rotavirus and adenovirus (EIA), calicivirus (RT-PCR), astrovirus (RT-PCR and EIA)	Virus identified in 48.5% and dual infection in 12% of cases. Of all cases: rotavirus 25.8%, calicivirus 24.2%, astrovirus 7.6%, adenovirus 3.0%. Rotavirus and norovirus peaked in the cold months of the year

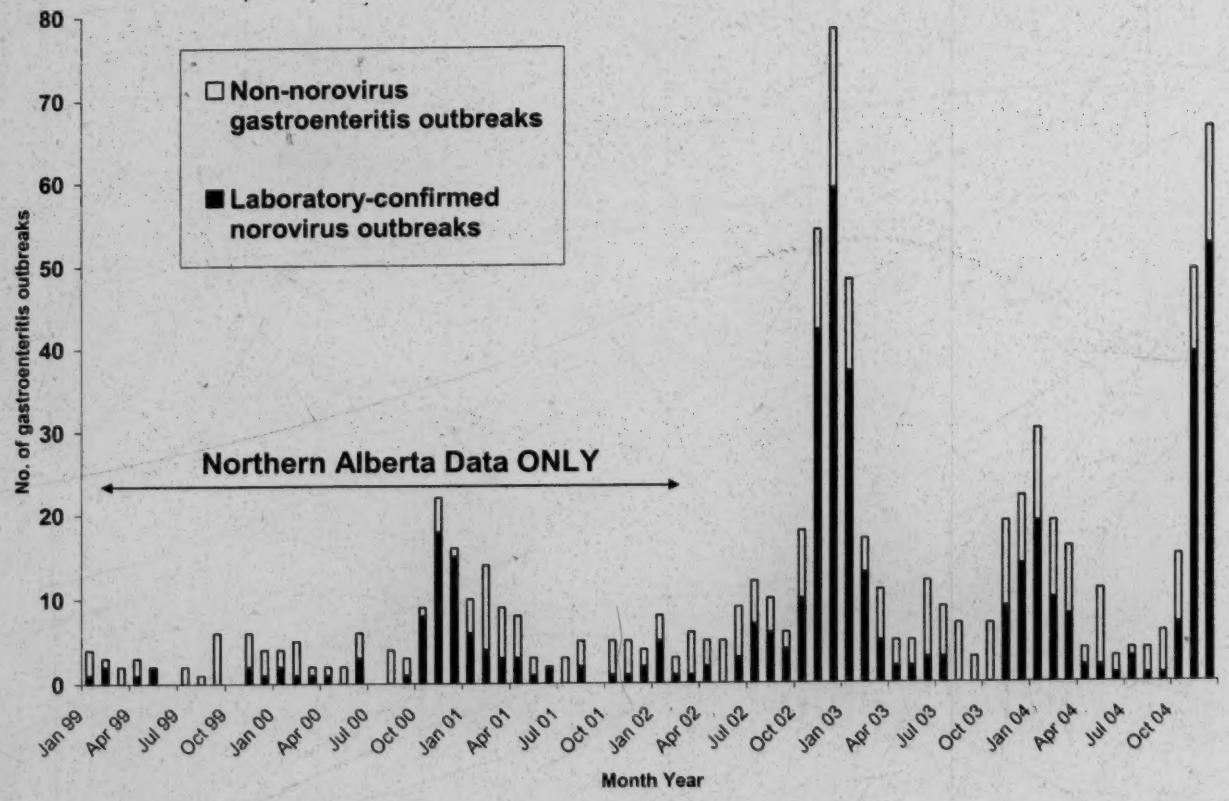
Chikhi-Brachet R[100]	2002	J Clin Micro	Case-control children and adults presented to General practice (sentinel network) with diarrhea in France (6 months Dec-May) (N=161 cases, N=45 controls)	≥3 soft or aqueous stool per 24 hours for <2 weeks	Rotavirus (EIA), norovirus & sapovirus (RT-PCR), Adenovirus (EIA), Astrovirus (EIA screen & RT-PCR confirmation)	Virus identified in 39.1% and dual infection 0.04% of cases. Of all cases: norovirus and sapovirus 19.2%, rotavirus 17.4%, astrovirus 4.3%, adenovirus 2.5%. 6.7% positive for virus in control: 2 rotavirus and 1 astrovirus. Six of 7 dual infection has norovirus, 3 has rotavirus. In terms of age: 54.4% positive in 0-3 years, 45.5% 4-15 years, 32.6% 16-65 years.
Subeklti D[119]	2002	FEMS Immun Med Micro	Case controls study with patients seen or admitted with diarrhea in	≥3 loose stool in preceding 24-72 hours	Rotavirus (EIA), norovirus (RT-PCR), Adenovirus	Rotavirus 42.3% (170/402) in cases, 7% in control; norovirus 20.6% (45/218 cases), 0% in control; adenovirus 4% (11/273

			Jakarta, Indonesia (21 month) (N=402 cases, N=70 controls, not all cases tested for all viruses)		(EIA), Astrovirus (EIA screen & RT-PCR confirmation)	cases), 0% in control. Rotavirus: 54% in 0-1 year, 35% 1-5 year. Norovirus 23.6% 0-1 year, 25.5% 1-5 year. Rotavirus peak in Jun and Jul, norovirus in Aug and Sept.
O'Neill HJ[102]	2002	J Clin Virol	Specimens submitted to Regional Virus Laboratory in Belfast, UK. (12 months) (N=1,945)	Not specified	EM and Multiplex PCR for adenovirus, rotavirus and norovirus	Virus identified in 21.5% and dual infection in 1% of cases. Of all specimens: rotavirus 9.7%, norovirus 6.7%, adenovirus 5%. In terms of age: rotavirus: 72% in 0-2 year, 27% 2-8 year. Norovirus: 32% 0-2 year, 11% 2-7 year, adenovirus: 74% 0-2 year, 24% 2-10 year. Rotavirus peak in Mar, Apr and May, norovirus in Jan and Feb.

Iritani N[27]	2003	J Clin Micro	Samples submitted from children <12 years presented to Sentinel clinics in Osaka, Japan (48 months) (N=669) 78% of samples from <3 years	Not specified	EIA for rotavirus and adenovirus, RT-PCR for norovirus and EM	Of all cases: rotavirus 28.2%, norovirus 15.6%, adenovirus 2.5%, astrovirus 0.1%. For norovirus, 91.4% were <3 years old. Norovirus occurs throughtout the year but 82.9% from Nov to Jan (Autumn to Winter)
Simpson R[101]	2003	J Med Virol	0-5 years inpatients and out-patients seen at Addenbrooke's Hospital in Cambridge, UK (12 months over 1999-2002: Oct 99 to Mar 00,	Not specified	EIA for rotavirus and adenovirus as well as RT-PCR for rotavirus, astrovirus and norovirus and DNA PCR for	Virus identified in 60.3% and 8% dual infection. Of all cases: rotavirus 34.4%, norovirus 18.7%, adenovirus 12.1%, astrovirus 3.0%, sapovirus 2.6%. Of the mixed infections, rotavirus was most common followed by norovirus

			Dec 00 to Feb 01, Dec 01 to Feb 02) (N=305)		adenovirus	
Oh DY[104]	2003	J Med Virol	Case controls: 19 days to 15.5 years inpatients seen at Berlin Charite Children's Hospital in Berlin, Germany (12 months) (N=217 cases, 50=control)	Not specified	Nested RT-PCR for rotavirus, astrovirus and norovirus and nested DNA PCR for adenovirus	Virus identified in 59% and 18% dual infection. Of all cases: rotavirus 47%, norovirus 21%, adenovirus 8%, astrovirus 2%. Of the mixed infections, rotavirus was most common followed by norovirus Of all controls: 5 (10%) positive for rotavirus and 3 (6%) positive for adenovirus, 2 (3%) for norovirus
Froggatt PC[105]	2004	J Med Virol	Samples submitted from children <7 years	Not specified	EM and EIA and RT-PCR for norovirus	Virus identified in 37.7% and 3.9% dual infection. Of all cases: rotavirus 22.7%,

			with sporadic diarrhea to Bristol Public Health Laboratory in Bristol, UK (6 months: Nov 99 to Apr 00) (N=3,172)			norovirus 10.3%, adenovirus 4.1%, astrovirus 3.2%. Of the mixed infections, norovirus was most common, followed by rotavirus. Norovirus most common in Jan and Feb; Mark of rotavirus in Mar. In terms of age, norovirus most common in 1-2 years. EM detected only 9% of the norovirus positive cases.
Zintz C[120]	2005	Infect Gene Evolut	15 days to 4 years presented to 3 hospitals with gastroenteritis of <7 days duration in Cincinnati, Ohio; Norfolk,	Not specified	RT-PCR for norovirus	Of all cases: norovirus (8.5%). Median age of infected cases: 266 days with positive results only in <4 years old.

			Virginia; Oakland, California (N=1,840)			
Liu C[106]	2005	J Clin Virol	21 days to 5 years outpatients seen at Beijing Children Hospital with gastroenteritis in Beijing, China (4 months: Dec 00 to Mar 01) (N=100)	Three or more unformed stool in a 24-hour period	EIA for rotavirus, astrovirus and adenovirus. RT-PCR for rotavirus, astrovirus and norovirus	Virus identified in 67% and 9% dual infection. Of all cases: rotavirus 59%, astrovirus 8%, norovirus 6%, adenovirus 2%. Of the mixed infections, rotavirus was most common

Appendix J Annual variation of laboratory confirmed norovirus outbreaks in Northern and Southern Alberta 2002-2004